University of Southampton

Faculty of Life and Environmental Sciences

School of Ocean and Earth Sciences

The Regulation of Daily Activity and Feeding Rhythms of the Economically Impactful Lepidopteran Pest *Plutella xylostella*

by

Connor J Tyler

ORCID ID 0000-0002-9849-4074

Thesis for the degree of Doctor of Philosophy

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Abstract

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Life forms have evolved mechanisms to remain 'in time' with the daily cycles of the planet. These circadian rhythms are often maintained via highly conserved molecular components called circadian clocks. These clocks regulate numerous physiological responses to both abiotic and biotic factors that can determine the effectiveness of pest management strategies. The diamondback moth, *Plutella xylostella*, is arguably the most economically impactful and widespread lepidopteran pest, having achieved a cosmopolitan distribution, causing ~$5 billion worth of damage annually. *P. xylostella* specializes in feeding on Brassica host plants and cruciferous crops while adult moths spread to fresh fields in a seasonally expanding range. Unique glucosinolate sulfatase enzymes allow *P. xylostella* to neutralize the glucosinolate/myrosinase defence system, which their host plants employ in a rhythmic fashion. Given the widespread impact of daily timekeeping on both plant and insect physiology, it is important to understand how both *P. xylostella* adult activity and larval feeding are controlled by internal and environmental rhythms. Our results showed that daily locomotor activity rhythms of adult moths resulted from a combination of light-mediated repression and homeostatic control of activity levels with little reliance on the circadian clock. However, qPCR results demonstrated the presence of molecular circadian clock components in adults that were maintained into constant conditions. Larval feeding rhythms also showed signs of light-mediated suppression,

showing typical nocturnal feeding rhythms across conditions, however, they also exhibited circadian control under constant light conditions where they damped out gradually. This indicated circadian clock-controlled herbivory which when in-phase with host plants showed minimal feeding around dawn and maximal around dusk. Comparison of herbivory on in-phase versus out-of-phase leaves indicated that both the phase of the larval and host plant clocks helped determine recorded daily feeding rhythms. Moreover, similar circadian rhythms to adults were demonstrated for *P. xylostella* larval transcripts encoding core molecular clock components. In addition, the above-mentioned glucosinolate sulfatases were also shown to maintain relative expression at particular times of day in larvae, suggesting a mechanism underlying the timing of herbivory. Herbivory rhythms were also maintained across host plants, however, when clock and jasmonic acid mutant hosts were used, feeding was significantly altered. This demonstrates that the phase and rhythm of host plant clocks significantly affect *P. xylostella* feeding, potentially through defence volatile signalling. These findings have potential implications for the temporal control of reproduction, migration and crop damage by *P. xylostella* and may be useful for informing integrated pest management strategies.

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Research Thesis: Declaration of Authorship

Print name: Connor J Tyler

Title of thesis: The Regulation of Daily Activity and Feeding Rhythms of the Economically Impactful Lepidopteran Pest Plutella xylostella

I declare that this thesis and the work presented in it are my own and has been generated by me as the result of my own original research.

I confirm that:

1. This work was done wholly or mainly while in candidature for a research degree at this University;
2. Where any part of this thesis has previously been submitted for a degree or any other qualification at this University or any other institution, this has been clearly stated;
3. Where I have consulted the published work of others, this is always clearly attributed;
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6. Where the thesis is based on work done by myself jointly with others, I have made clear exactly what was done by others and what I have contributed myself;
7. None of this work has been published before submission

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Definitions and Abbreviations

Due to this project covering both animal and plant disciplines, there are inconsistences between the standard nomenclature used for describing the genetics of animal and plant genes and gene products. In plant genetics, wild-type genes are written in capitalized letters and italics, while mutants are written in lower case, with both protein products naming not italicized, following The Arabidopsis Information Resource guidelines. Within animal genetics there are also differences, with human gene nomenclature denoting genes in a similar fashion to plants, following guidelines noted in Bruford et al., 2020.

As the focus of this project is dedicated to *P. xylostella*, and how other pest species like it interact with environmental factors, insect gene and gene product nomenclature is used throughout, following the guidelines of FlyBase. This is to improve understanding of what gene and gene product names refer to within the thesis and increase ease of use. Gene symbols are italicized and begin with a capitalized letter, except those named from recessive mutations which begin with a lowercase letter, followed by all lowercase letters. Proteins are not italicized and always begin with a capital letter with capitalised symbol.

ALAN.....Artificial light at night

ANOVA .....Analysis of variance

aos/AOS .....Allene oxide synthase

Bmal1/BMAL1.....Brain and muscle ARNT-like 1

*Bt*T.....*Bacillus thuringiensis* toxin

CAB .....Chlorophyll a/b-binding protein

cca1/CCA1 .....Circadian clock associated 1

cca1-ox .....*circadian clock associated 1 – overexpressor*

CCN .....Central clock neurons

CHE.....CCA1 hiking expedition

CHS.....Chalcone synthase

CK1δ/ε .....Casein kinase 1 delta/epsilon

CKII .....Casein kinase 2

Clk/CLK.....Clock

Definitions and Abbreviations

coi1/COI1 ..... Coronatine-insensitive 1

coi1-16 ..... *Coronatine-insensitive 1 mutant*

CREB ..... Cyclic AMP-responsive element-binding protein 1

CRISPR-Cas9 ..... Clustered regularly interspaced short palindromic repeats and CRISPR-associated protein 9

(d/m)*cry*/CRY ..... (*Drosophila*-like/mammalian-like) Cryptochrome

CT ..... Circadian time

CWO ..... Clockwork orange

cyc/CYC ..... Cycle

d ..... Distilled

DAD1 ..... Defender against cell death 1

DAM ..... Drosophila activity monitor

DAMPs ..... Damage-associated molecular patterns

DBT ..... Doubletime

D/D ..... Constant dark

DDT ..... Dichlorodiphenyltrichloroethane

DEC1, 2 ..... Differentiated embryo chondrocyte 1, 2

dn-OPDA ..... Dinor-oxo phytodienoic acid

DPM ..... Drosophila population monitor

ECR ..... Environmental control rooms

Ef1α ..... *Elongation factor 1 α*

ELF3, 4 ..... Early flowering 3, 4

GAL4-UAS ..... Galactose-responsive transcription factor-upstream activating sequence

GI ..... Gigantea

GM ..... Genetically modified

gre1 ..... *Glyphosate response 1*

GSS ..... Glucosinolate sulfatase

GST ..... Glutathione-S-transferase

GSTu1 ..... Glutathione S-transferase u1

h..... Hour(s)

IQR..... Interquartile range

IPM..... Integrated pest management

JA ..... Jasmonic acid

JA-Ile..... Jasmonoyl isoleucine

JAR1..... Jarin-1

JAZ..... Jasmonate-zim domain

JET ..... Jetlag

LED..... Light emitting diode

LUC..... Luciferase

IP ..... In-phase

LC_{50}..... Lethal concentration 50

L/D ..... Light/Dark

lhy/LHY..... Late elongated hypocotyl

L/L..... Constant light

LUX ..... Lux arrhythmo

MAPK ..... Mitogen-activated protein kinase

MeJA ..... Methyl jasmonate

MOB..... Mustard oil bomb

OP..... Out-of-phase

OPC-8:0 ..... 3-oxo-2(2'-[Z]-pentenyl)cyclopentane-1-octanoic acid

12-OPDA ..... 12-Oxo phytodienoic acid

opr3/OPR3 ..... Oxophytodienoate-reductase 3

opr3-1..... *oxophytodienoate-reductase 3 mutant*

PAMPs ..... Pathogen-associated molecular patterns

PDP1 ..... PAR domain protein 1

PEP1 ..... Plant elicitor peptide 1

per/PER..... Period

PHT4;1..... Phosphate transporter 4;1

PHY ..... Phytochrome

PP2A..... Protein phosphatase 2A

PRR..... Pseudo-response regulators

*Px*ABCC2, 3 ..... *Plutella xylostella* ATP-binding cassette transporters C2, 3

PxGSS1, 2, 3 ..... *Plutella xylostella glucosinolate sulfatase 1, 2, 3*

PxSulfB, D..... *Plutella xylostella sulfatase B, D*

*Pxyl*CSP11 ..... *Plutella xylostella* Chemosensory protein 11

*Pxyl*Gr34 ..... *Plutella xylostella* gustatory receptor 34

PxylSulf2, 3, 4, 1/*Sulf 2, 3*.*Plutella xylostella sulfatase 2, 3, 4, 1*

PxylSumf1a ..... *Plutella xylostella sulfatase modifying factor 1 alpha*

qPCR..... Quantitative polymerase chain reaction

REV-ERBα ..... Nuclear receptor subfamily 1 group D member 1 alpha

RORα..... RAR-related orphan receptor alpha

RRP..... Relative rhythmic power

s..... Subjective

SIK1 ..... Salt inducible kinase 1

SCN..... Suprachiasmatic nucleus

SGG ..... Shaggy

si-Pxace1..... Small interfering *Plutella xylostella* angiotensin converting enzyme I

siRNA ..... Small interfering RNA

SIT ..... Sterile insect technique

Sulf-C ..... *Sulfatase C*

SUMF1 ..... Sulfatase modifying factor 1

ST2A ..... Sulfotransferase 2A

tim/TIM ..... Timeless

TM ..... Triple mutant / *cca1-11/lhy-21/toc1-21*

toc1/TOC1 ..... Timing of CAB expression 1

TTFL ..... Transcription and translation feedback loops

UoS ..... University of Southampton

VRI ..... Vrille

WT ..... Wild type

xct ..... *XAP5 circadian timekeeper*

ZT ..... Zeitgeber time

ZTL ..... Zeitlupe

Chapter 1 Literature Review

1.1 Abstract

Circadian rhythms are shown across numerous aspects of physiology as life attempts to stay in time with the daily cycles of the planet. Conserved mechanisms to maintain biological rhythms have also arisen, with the use of circadian clocks and precise timing shown across life. Therefore, the harnessing of rhythms in physiology offers potentially exploitable pathways to increase the effectiveness of pest management strategies as climate change and a growing human population increase pressure on agricultural systems, while pest and pesticide damage increases in severity. Research has widely shown daily time-dependent effects of pest treatments, also known as chronotoxicity, and have found that increasing overall quantities of chemicals used in treatments do not overcome these time-sensitive responses. Yet research has also shown high levels of variation and species-specific effects, potentially making the study and application of circadian rhythms more difficult. However, advancements in technology and chronobiology research may allow for the integration of circadian rhythms with pest management strategies, potentially increasing their efficacy and allowing for wider adoption, reducing costs and environmental damage. The lepidopteran order contains many economically impactful pests but perhaps none more so than the Diamondback Moth (*Plutella xylostella*), therefore making *P. xylostella* a prime candidate for understanding how they cause damage and whether circadian rhythms may be harnessed to reduce such. *P. xylostella* is a brassica feeding specialist that seeks out the secondary metabolite glucosinolate, which is rhythmically produced by studied Brassicaceae species and broken down by a specialised protein unique to *P. xylostella*. *P. xylostella* uses glucosinolate as a cue to locate its preferred plant hosts, where, by overcoming this metabolite, it may have a competitive advantage over other insect herbivores. The lepidopteran circadian clock may be involved

in the regulation of numerous rhythmic physiological processes including breeding, feeding and detoxification, potentially including that of glucosinolates. Due to the species-specific nature of circadian rhythms and chronotoxicity, these rhythmic processes need to be investigated directly in *P. xylostella* as well as the interactions with host plants, which have also been shown to affect the aforementioned factors. Such findings may help increase the efficacy of, or even provide novel, integrated pest management strategies to control *P. xylostella* pest populations.

1.2 On the clock! How circadian rhythms affect pest management

1.2.1 Introduction to circadian rhythms

1.2.1.1 Definition of circadian rhythms

Life forms have evolved mechanisms that allow organisms to be in time with the roughly 24-hour (h) daily cycles of the planet. These subsequent daily rhythms in physiology can be maintained internally (endogenous), without the need for external stimuli, while being able to be entrained or compensate for such to achieve rhythmic activity. Activity that matches these criteria can be said to have circadian rhythms (Pittendrigh, 1960). Circadian rhythms are a crucial part of species' adaption to the world around them, allowing for genes and behaviours to be differentially expressed across the day to best suit an organisms needs, including the anticipation of daily changes which can help to further increase a species' fitness (Paranjpe and Sharma, 2005, Vaze and Sharma, 2013, Xu et al., 2022). The importance of such for fitness is exemplified by circadian rhythms highly conserved nature across the domains of life (Edgar et al., 2012), found everywhere from bacteria (Sartor et al., 2019, Eelderink-Chen et al., 2021) to human brain function (Pantazopoulos et al., 2018, Logan and McClung, 2019). These rhythms are maintained by the circadian clock, of which certain genetic components are also highly conserved within kingdoms, being able to be tracked across evolutionary time (Dunlap, 1999, Nawathean, Stoleru and Rosbash,

2007, Romanowski et al., 2014). A model of function through interconnected positive and negative transcription and translation feedback loops (TTFL) has also been found to be crucial to circadian clocks across most life (Hurley, Loros and Dunlap, 2016, Dunlap and Loros, 2017). The ubiquitousness of the circadian clock and subsequently controlled rhythms point to its utmost importance to organism's fitness.

1.2.1.2 Biological functions of circadian rhythms

The natural world has many cycles in abiotic and subsequently biotic factors which it can be advantageous for organisms to be in sync with. Perhaps the most prominent cycle being that of night and day. The key change between these two conditions is the presence of light, which acts as a powerful zeitgeber (time giver) that can also directly cause cycles in other factors such as temperature, another zeitgeber species can use to entrain circadian rhythms (Boothroyd et al., 2007, Tessmar-Raible, Raible and Arboleda, 2011, Schmal, Herzel and Myung, 2020). These zeitgebers entrain the circadian clock by gating the expression of clock genes as they change over time, leading to up and down-regulation of genes at specific points across the 24h day. Species can use numerous signals as zeitgebers from light colour (Krull, 1976, Vetter et al., 2011, Pauers et al., 2012) and feeding (Prendergast et al., 2015, Cisse et al., 2018) to stress (Pellman et al., 2015, Pierre et al., 2018) and social interactions (Mistlberger and Skene, 2004, Fuchikawa et al., 2016). Combinations of such zeitgebers may also have combinatory or competing interactions, including between tissue specific circadian clocks (Peripheral clocks) (Giebultowicz, 2001, Mohawk et al., 2013, Grabe et al., 2022). Light signals are often perceived as the most dominant zeitgebers (Hau and Gwinner, 1997, Roenneberg and Merrow, 2000, Schmal C, Herzel H and Myung J, 2020), likely due to its interaction with other abiotic factors and organisms' exposure to predation and food (Prugh and Golden, 2014, Bennie et al., 2018). Specific factors also may have comparatively more dominant effects on defining circadian rhythms and in different parts of the body, showing evidence of further levels of plasticity

to maintained circadian rhythms (Abraham et al., 2010, Dunlap and Loros, 2017). Responding to these environmental fluctuations and prioritising those factors that have the greatest effects on fitness and survival allows species to best adapt to their environment. Responses to zeitgebers can also have high sensitivity, readily altering rhythmic responses (Brown et al., 2002, Vinayak et al., 2013, Philips et al., 2019), further demonstrating the importance of maintaining circadian rhythms and regulating such in response to even subtle environmental cues. Having endogenous circadian rhythms that match the environment significantly increases fitness (Horn et al., 2019), additionally, individuals that remain in constant environments may still be entrained to natural cycles by harnessing cues from interactions with others, such as young bees (*Apis mellifera*) that remain inside the hive (Fuchikawa et al., 2016, Siehler, Wang and Bloch, 2021). Maintenance of entrained rhythms across generations lacking any zeitgebers has also been shown, even increasing in rhythm robustness (Shindey et al., 2016). Such findings help to highlight the important role rhythmic entrainment holds in species adaptations to the specific environments they live in. The key benefit for maintaining rhythmic physiology entrained by zeitgebers, over directly responding to changes in diel environmental factors or resetting a daily timer, is the anticipation of environmental changes and threats.

Anticipation of daily changes and threats is the primary way species increase their fitness using circadian rhythms. Predicting changes in environmental factors allows species to pre-emptively organise expression of genes and behaviours to meet the needs of a fluctuating environment (Vaze and Sharma, 2013), specialising to the exploitation or aversion of specific conditions. Variations in factors can therefore make environments more or less hospitable to species adapted to such conditions occurring at certain times of the day. These preferred environments can be called temporal niches (Refinetti, 2007, Hut et al., 2012). Numerous negative effects can occur when species are out of sync with entrained cycles due to the inappropriate allocation of resources to time specific activity (Dodd et al.,

2005, Bass, 2012, Cisse et al., 2018), with there often being significant reductions in survival and fitness when organisms are deprived of their temporal niches. Such as with increases in light pollution, disrupting numerous species behaviours, including threatened amphibian species (Penev et al., 1998, Desouhant et al., 2019, Touzot et al., 2019, Vinne et al., 2019, Boyes et al., 2020, Touzot et al., 2020). Due to the strong selection to specific temporal conditions, organisms between and of the same species that begin to occupy varying temporal niches, also helping to avoid competition (Schwinning and Kelly, 2013, Lear et al., 2021), can quickly become temporally isolated without the need for environmental changes, leading to speciation events and facilitating rapid adaptive radiation (Tan, Kelly and Jiang, 2013, Boumans et al., 2017). Taking these factors into account, the genes responsible for the maintenance of circadian rhythms are therefore highly selected for with highly plastic downstream regulatory pathways to facilitate specialisation to varying environmental conditions.

1.2.1.3 Molecular organisation of circadian clocks

Circadian clocks are found across the kingdoms of life, however, the exact makeup of such can differ greatly, with novel genetic components found maintaining circadian rhythms within different phyla (Dunlap, 1999). Circadian rhythms were first recorded scientifically centuries ago (d'Ortous, 1729) and by the 1970s the first molecular components were also described. The first discovery was of the *period* (*per*) gene, after Konopka and Benzer demonstrated mutations in a previously unknown gene caused disruptions to the circadian clock in fruit flies (*Drosophila melanogaster*) (Konopka and Benzer, 1971, Bargiello, Jackson and Young, 1984). Since then, research on several model organisms has been crucial in the understanding of the molecular clock, showing many examples of unique molecular architecture. For example using *Arabidopsis thaliana*, a brassica species, for plants (McClung, 2006) and *Neurospora crassa*, a type of bread mould, for fungi (Baker, Loros and Dunlap, 2012). Animal models too show specific differences, including between

D. melanogaster, for arthropods (Rosato, Tauber and Kyriacou, 2006) and *Mus musculus*, house mice, for mammals (Chang and Reppert, 2001). Zebra fish (*Danio rerio*) also provided an important complimentary model clock for understanding vertebrate clock systems (Steinland and Whitmore, 2019). In spite of their differences, these clocks are able to maintain endogenous rhythms in line with the proposed qualifiers of true circadian rhythms, unified by relying on a TTFL model and core molecular oscillating components (Pittendrigh, 1960, Saini, Jaskolski and Davis, 2019).

While organisms across phyla possess the roughly 24h circadian clock fitting the TTFL model, the molecular components regulating the plant clock are genetically unrelated to those in the animal kingdom. The TTFL model applies to the core plant molecular circadian oscillator components, made up of the transcription factor genes *cca1*, *lhy* and *toc1* (*circadian clock associated 1, late elongated hypocotyl, timing of CAB expression 1*), each involved in interconnected feedback loops. This clock system shown in Fig. 1.1 can be entrained by photoreceptors that absorb both blue and red light, using cryptochromes (CRYs) and phytochromes (PHYs) respectively. Without the presence of light TOC1 is degraded and cannot positively regulate *cca1* and *lhy* expression, normally expressed at dawn. TOC1 is protected from degradation upon light perception by CRY and PHY, allowing *cca1* and *lhy* to be expressed. However, CCA1 and LHY inhibit *toc1* expression, completing this simplified version of the basic negative feedback loops in plants core circadian oscillator (Gardner, et al., 2006, McClung, 2006). The circadian clock endogenous period, shown through constant condition experiments appeared to be dependent on both age of *A. thaliana* leaf tissues (~22.5h in older leaves vs ~24h in younger) (Kim et al., 2016), with tissue specific clocks also found (Thain et al., 2002, Endo et al., 2014, Shimizu et al., 2015). Differing plant species can have varying periods also such as *Brassica rapa* (Field mustard), having a longer period of ~25h. (Xu, Xie and McClung, 2010), including between peripheral clocks, with opposite differences between

root and shoot clocks to *A. thaliana* found in *Medicago trunctula* (barrelclover), a legume (James et al., 2008, Wang et al., 2021).

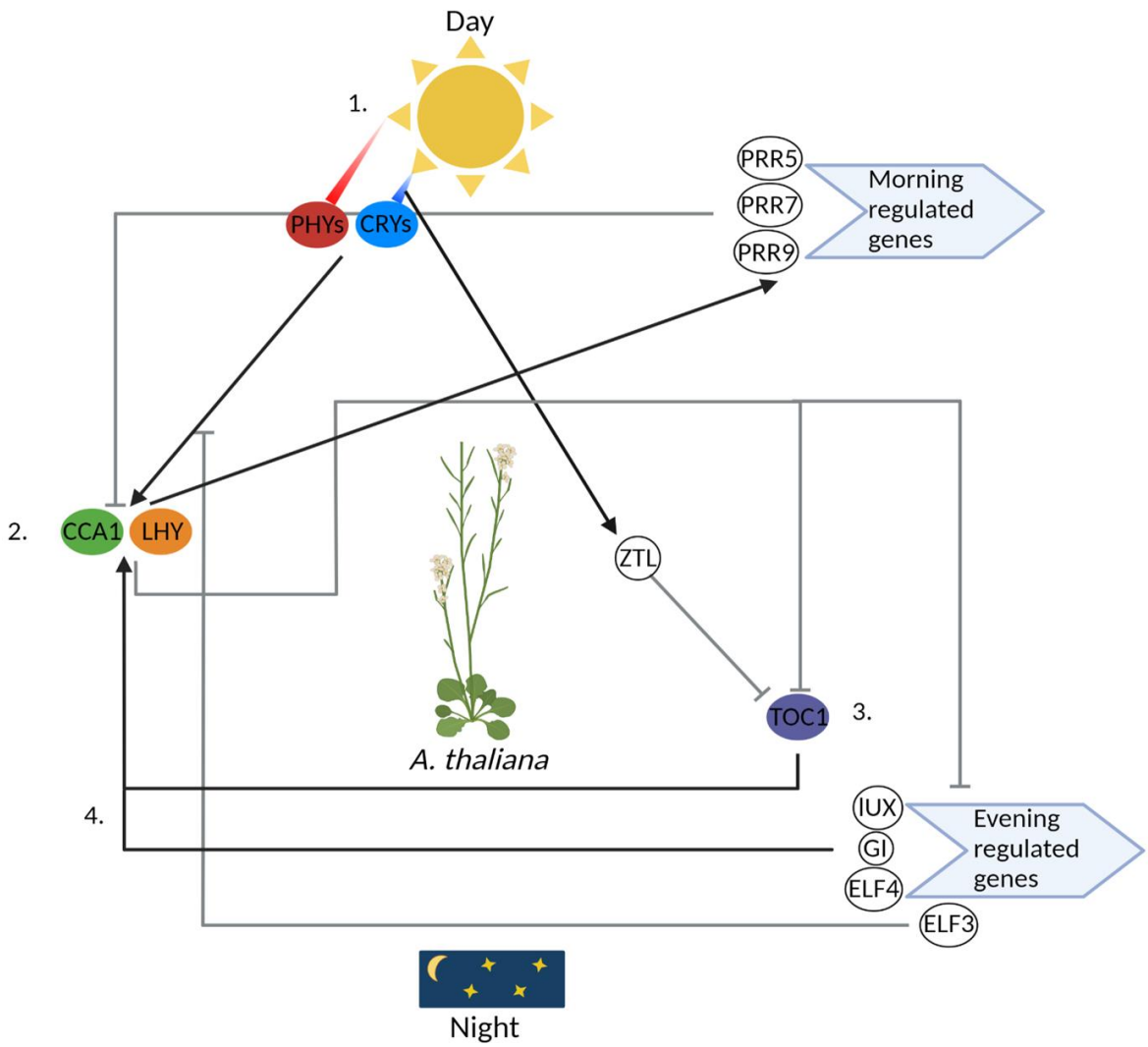


Figure 1.1 Plant (*A. thaliana*) model molecular circadian clock

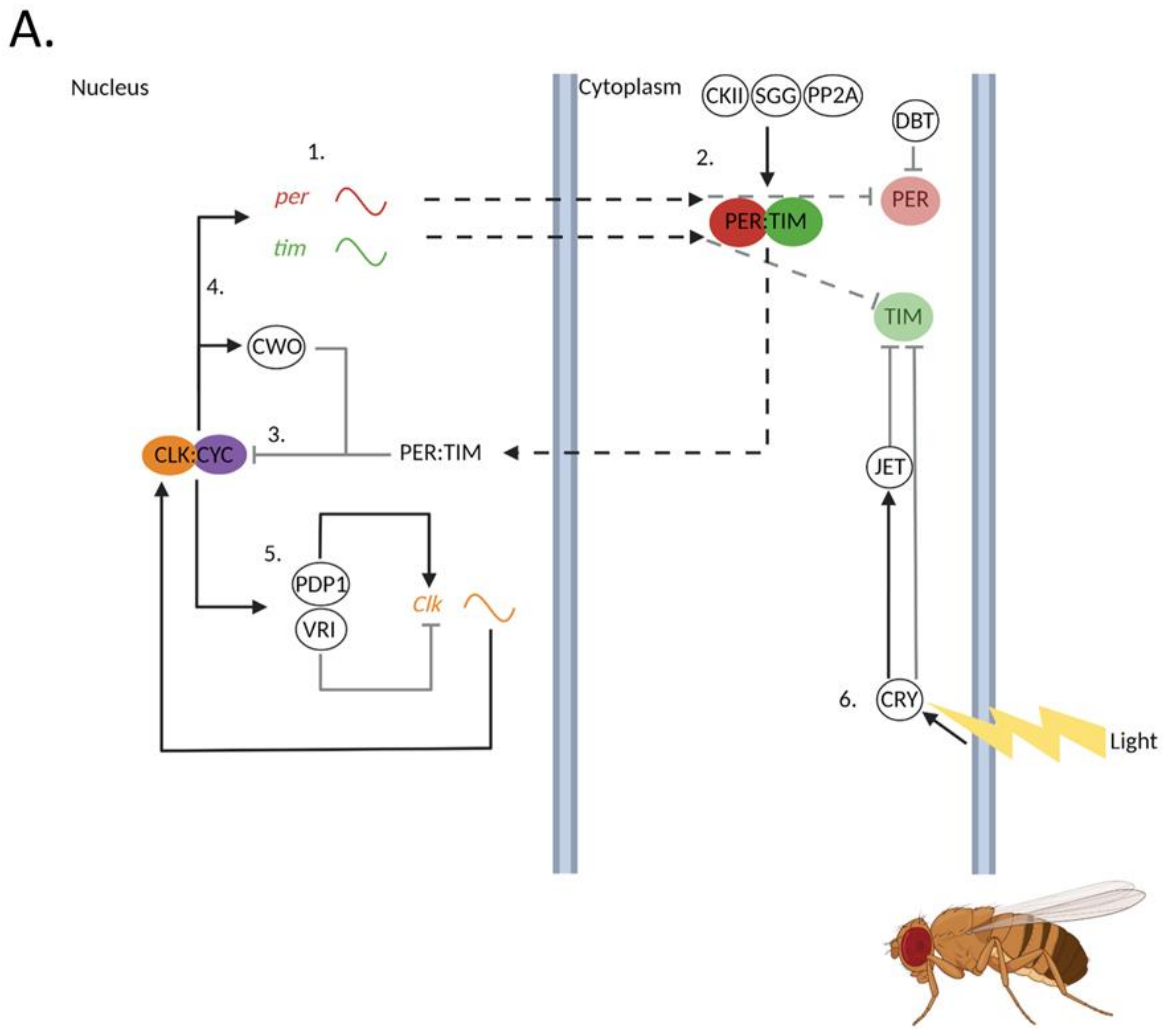
Solid black arrows equate to promotion, flat head grey arrows indicate inhibition and hashed arrows equate to molecular movement specifically. Lower case italics indicate genes with lines indicating transcripts next to such, upper case in circles indicates proteins with joined proteins indicating complexes. Colour coding for genes and circles for proteins are also used to highlight key rhythmic circadian clock genes. Faded boxes also show degradation of proteins.

The plant circadian clock is predominantly modelled using *A. thaliana.* 1. Sunlight activates both blue light (CRYs) and red light (PHYs) photoreceptors. 2. In the morning, signals from the activation of both CRYs and PHYs promote the accumulation of Ca^{2+} and the production of CCA1 and LHY (Martinez-Garcia, Huq

and Quail, 2000, Xu et al., 2007). Both CCA1 and LHY then go onto upregulate morning genes, some of which produce transcription factors (Pseudo-response regulators (PRR) 5, 7 and 9) that then act to repress their own production (Nakamichi et al., 2010, Nagel et al., 2015), with both CCA1 and LHY shown to be crucial for defence and development regulation, often through jasmonic acid signalling (Goodspeed et al., 2013a, Nitschke et al., 2016, Zhang, Bo and Wang, 2019). 3. TOC1 production is repressed by CCA1 and LHY along with repression of evening genes (Alabadi et al., 2001, Kamioka et al., 2016). ZTL (Zeitlupe), a protein that usually represses TOC1, is also a blue light receptor and it's accumulation peaks at evening (Kim, Geng and Somers, 2003, Mas et al., 2003, Fujiwrara et al., 2008). 4. Both TOC1 and a selection of transcription factors (Lux arrhythmo (LUX), Gigantea (GI), Early flowering 4 (ELF4)) produced by evening genes promote the production of CCA1 and TOC1 (Park et al., 1999, Doyle et al., 2002, Hazen et al., 2005), however ELF3, another protein that accumulates in the evening, down regulates CCA1 and TOC1 production by inhibiting light mediated promotion of such (Kikis, Khanna and Quail, 2005).

The *D. melanogaster* clock, the standard model for arthropods, shows a similar negative feedback loop in the core molecular clock following the TTFL model, shown in Fig. 1.2A. The *per* gene was the first discovered, being successfully cloned, characterised and used to rescue clock function in *D. melanogaster* mutants in the 1980s (Bargiello and Young, 1984, Bargiello, Jackson and Young, 1984, Zehring et al., 1984), however, over the decades the model of the core molecular clock has increased in complexity. It has been shown that PER is joined by TIM in the cytoplasm where they undergo post-translation modifications to form a heterodimer that can undergo nucleus entry where it subsequently represses both *per* and *tim* expression. This occurs via interaction with the CLK:CYC heterodimer which would otherwise promote *per* and *tim* expression (Hardin, 2005). CLK and CYC are themselves part of another negative feedback loop as well as repressing their own activity via the promotion of an additional inhibitor (Cyran et al., 2003, Richier et al., 2008). *D. melanogaster* maintains an endogenous period length of ~24h, however this can be significantly altered with numerous clock mutants (Konopka and Benzer, 1971,

Hamblen-Coyle et al., 1992). Other arthropod molecular clocks are also being uncovered with reviews showing variations in the number of known clock components involved to maintain circadian rhythmicity in specific species (Kotwica-Rolinska et al., 2021) with insect groups such as Lepidoptera, showing further similarities with known mammalian molecular circadian clock architecture (Brady et al., 2021).



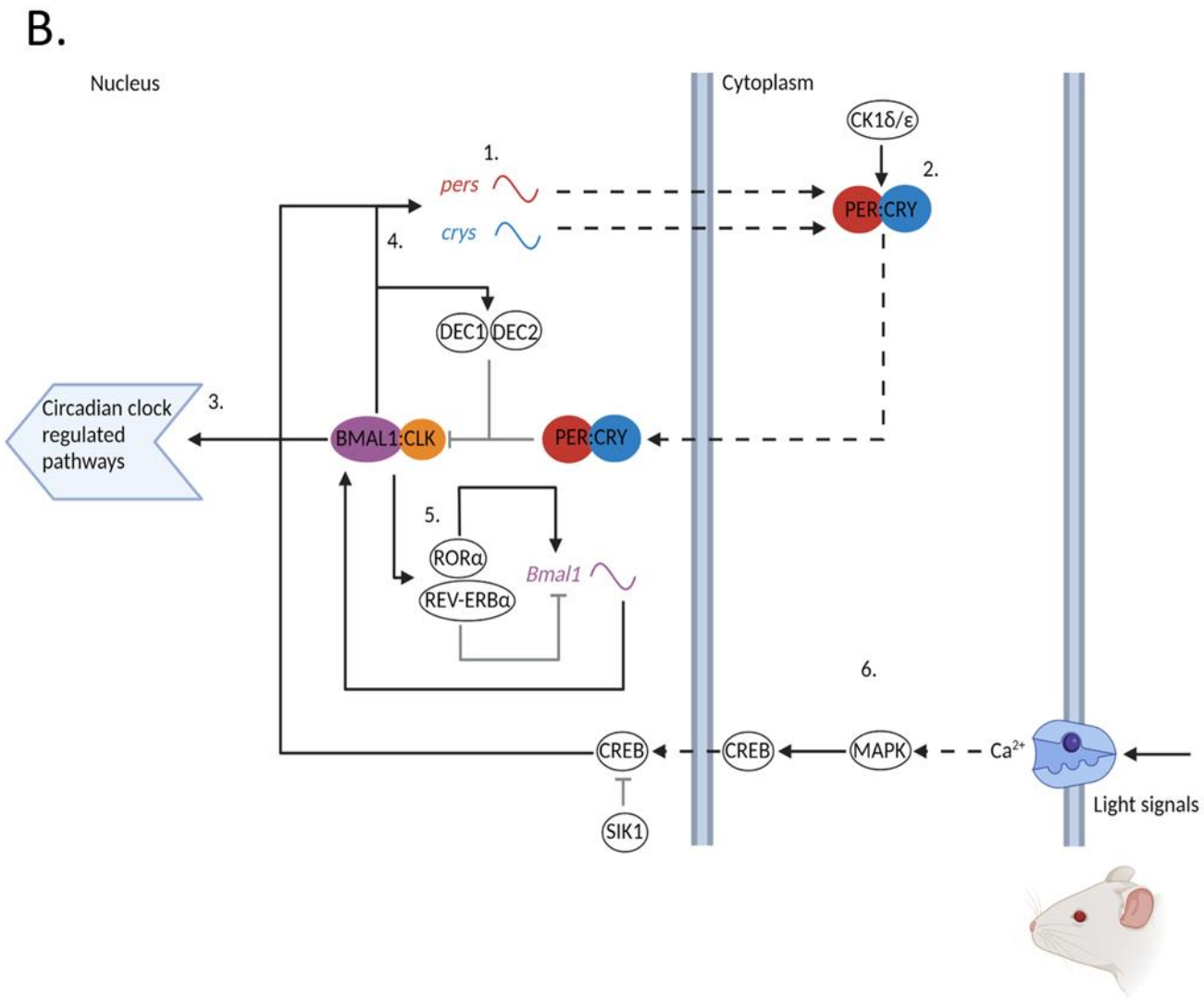


Figure 1.2 The model animal molecular circadian clocks of A. Fruit fly (*D. melanogaster*) and B. Mice (*M. musculus*)

Solid black arrows equate to promotion, flat head grey arrows indicate inhibition and hashed arrows equate to molecular movement specifically. Lower case italics indicate genes with lines indicating transcripts next to such, upper case in circles indicates proteins with joined proteins indicating complexes. Colour coding for genes and circles for proteins are also used to highlight key rhythmic circadian clock genes. Faded boxes also show degradation of proteins.

A. The insect molecular circadian clock is predominantly modelled using *D. melanogaster*. 1. PER and TIM (Timeless) is produced and accumulates in the cytoplasm as part of the core circadian molecular oscillator (Konopka and Benzer, 1971, Sehgal et al., 1994, Rosato, Tauber and Kyriacou, 2006). 2. A PER:TIM complex is formed, stabilised by CKII (Casein kinase 2), SGG (Shaggy) and PP2A (Protein phosphatase 2A) to allow for nucleus entry (Martinek et al., 2001, Akten et

al., 2003, Sathyanarayanan et al., 2004). However, DBT (Doubletime) promotes the degradation of PER (Price et al., 1998) and JET (Jetlag) for TIM (Koh, Zheng and Sehgal, 2005). 3. After nucleus entry the PER:TIM complex inhibits the CLK (Clock):CYC (Cycle) complex (Glossop, Lyons and Hardin, 1999). 4. The CLK:CYC complex otherwise promotes the production of PER and TIM and therefore completes the 1st core negative feedback loop with both complexes affected interacting with circadian clock regulated pathways (Darlington et al., 1998, Lee, Bae and Edery, 1999). An additional transcription factor is produced (Clockwork orange (CWO)) that also inhibits its own promoter, creating another negative feedback loop against CLK:CYC function (Kadener et al., 2007, Richier et al., 2008). 5. The CLK:CYC complex also promotes PDP1 (PAR domain protein 1) and VRI (Vrille) transcription factors in a secondary negative feedback loop, however both act on *Clk* with PDP1 being a promoter while VRI is an inhibiter (Cyran et al., 2003). 6. A photoreceptive CRY is activated by light and interacts with TIM to trigger degradation, also recruiting JET for such, therefore inhibiting formation of the PER:TIM complex (Emery et al., 1998, Berntsson et al., 2019).

B. The mammalian molecular circadian clock is predominantly modelled using *M. musculus.* 1. Both CRY and PER proteins have circadian rhythms in production and have been shown to be key parts of the core circadian molecular oscillator, however, unlike with *Drosophila*-like (d)CRY and dPER, mammalian-like (m)PER is shown to have 3 distinct genes along with both mCRYs being non-photoreceptive (Tei et al., 1997, Griffin, Staknis and Weitz, 1999, Kume et al., 1999). 2. Once translated in the cytoplasm PER and CRY form a complex that is stabilised by phosphorylation, allowing nucleus entry for the complex. CK1δ/ε (Casein kinase 1, delta/epsilon) interactions facilitates this with this function conserved between *D. melanogaster* (CKII) and *M. musculus* (Kume et al., 1999, Lee et al., 2001, An et al., 2022). 3. The PER:CRY complex then represses BMAL1 (Brain and muscle ARNT-like 1):CLK complex activity, this complex also being responsible for the regulation of many circadian clock regulated pathways (Panda et al., 2002, Trott and Menet, 2018). 4. This creates the core negative feedback loop as this complex also promotes PER and CRY production (Sherman et al., 2000, Sato et al., 2006) in addition to DEC (Differentiated embryo chondrocyte) 1 and 2 transcription factors that also inhibit BMAL1:CLK (Honma et al., 2002). 5. An additional negative feedback loop also exists between the

BMAL1:CLK complex and RORα (RAR-related orphan receptor alpha) and REV-ERBα (Nuclear receptor subfamily 1 group D member 1 alpha). BMAL1:CLK promotes the production of both, however while the transcription factor RORα also promotes BMAL1, REV-ERBα inhibits its production, forming another negative feedback loop (Sato et al., 2004, Triqueneaux et al., 2004). 6. Movement of calcium ions into the cytoplasm in response to light input signals activates a number of pathways. This includes the activation of MAPK (Mitogen-activated protein kinase) that allows for CREB (Cyclic AMP-responsive element-binding protein 1) entry into the nucleus where such can entrain the molecular circadian clock (Obrietan, Impey and Storm, 1998, Dolmetsch et al., 2001, Lee et al., 2012). SIK1 (Salt inducible kinase 1) can repress CREB, limiting light entrainment in mammals (Jagnnath et al., 2013).

A series of interconnected negative feedback loops is also seen in the mammalian clock along with a number of clock component homologues, shown in Fig. 1.2B. Shown from studies on *M. musculus*, the mammalian clock shares some similar molecular components, however the most notable difference is the loss of *tim* from the core circadian oscillator, being replaced by a cryptochrome (mCry), along with multiple *per* genes being present and *cyc* renamed to *Bmal1* (Rosato, Tauber and Kyriacou, 2006, Kwon et al., 2011, Buhr and Takahashi JS, 2013). In comparison to mCry, dCry acts as the key photosensitive pathway in the *D. melanogaster* molecular clock, entraining the core circadian oscillator to the presence of light, however dCRY appears to not be as evolutionarily old as mCry (Zeng et al., 1996, Dubowy and Sehgal, 2017). Mammalian period lengths can have higher degrees of variation, however *M. musculus* showed ~23.5h (Wee et al., 2002, Brown et al., 2005, Pagani et al., 2010).

1.2.1.4 Circadian regulation

After using photoreceptive and other zeitgeber pathways to entrain circadian clocks, numerous downstream pathways can regulate physiological systems (Paula et al., 2008, Ikeno et al., 2010, Adams and Carre, 2011). Circadian rhythms have widespread effects on gene expression and signalling (Takahashi, 2016), with ~35% of the total plant

transcriptome under circadian regulation (Michael and McClung, 2003) and ~40% of coding genes in *M. musculus* expressing rhythmically (Zhang et al., 2014). This control can be hierarchical however, with central clock neurons (CCN) communicating with various peripheral clocks to keep organs and tissues in time with the rest of the body (Albrecht, 2012, Selcho et al., 2017, Honma, 2018), with peripheral clocks able to interact with each other further (Endo et al., 2014). Physiological shifts in organ function can rely on the interplay between CCN signals and peripheral clocks (Bajgar, Jindra and Dolezel, 2013), with peripheral clocks also being shown to entrain to environmental conditions without the need for CCN or in some cases any connected sensory organs (Giebultowicz, 2001, Nobata et al., 2012). However, tissue-specific circadian clocks can maintain specific circadian rhythms long-term and can drift out-of-phase (OP) with each other (Mohawk, Green and Takahashi, 2012, Merrow and Harrington, 2020). Nonetheless, the ubiquitous presence of circadian rhythms throughout organism's bodies demonstrates the importance of maintaining precisely timed expression and activity, also providing predictable variations in such, with mismatched rhythms causing negative effects to species fitness (Takeda and Maemura, 2011, Goodspeed et al., 2012, Bass, 2012, Cisse et al., 2018).

1.2.1.5 Pest management and circadian rhythms

Circadian rhythms were first highlighted to play a part in an organism's response to pest management efforts in the 1960s, showing how over the 24h day *Blattella germanica* had varying resistance to pesticide (Beck, 1963). Numerous following studies have shown similar effects across multiple clades of life (Shipp and Ottom, 1976, Miller et al., 2002, Tkadlec and Gattermann, 2008), demonstrating how the rhythmic regulation of species' immune and detoxification responses may provide useful information to improve the efficacy of pest control treatments (Hooven et al., 2009). This may apply to potentially environmental damaging pesticides (Gill and Garg, 2014) but also to various treatments in integrated pest management (IPM) strategy. The use of IPM aims to reduce the now widely

appreciated negative effects of broad-spectrum pesticide treatments in agriculture. Towards this aim multiple different avenues of research have been harnessed to help control pest species populations through specific targeted chemical treatments, baiting, natural enemy provision and more (Kogan, 1998, Ehler, 2006, Wang and Bennet, 2006), with modern advancement in genetics allowing for the release of genetically modified (GM) conspecifics with deleterious traits (Dunn and Follett, 2017). These varying management pathways may benefit from the precise timing of treatments due to the previously mentioned variations in species physiology, helping to increase the efficacy and therefore economic viability of these less environmentally damaging treatment options.

1.2.2 Physiology under circadian regulation

1.2.2.1 Central circadian system

Plant species have a more diffuse circadian clock network, lacking a central nervous system, however, they appear to have tissue-specific clocks regulating physiological rhythms with each cell maintaining circadian rhythmicity (Endo et al., 2014, Shimizu et al., 2015, Endo, 2016, Creux and Harmer, 2019). Animal species posses more centralised circadian clock regulation, with specific CCN located in the central nervous system that can inform tissues throughout the body (Mohawk, Green and Takahashi, 2012,). In *D. melanogaster*, clock cells have been identified in the brain that communicate between each other to maintain circadian rhythms in activity, with loss of function/rhythms in subsets of either lateral or dorsal clock neurons altering rhythmic outputs (Grima et al., 2004, Stoleru et al., 2004, Reinhard et al., 2022). These CCN can receive information on light through optical pathways relying on cells in the retina to transmit signals informing sleep and wake rhythms (Helfrich-Forster, 2019, Damulewicz et al., 2020). However, these optical cells may also possess peripheral clocks and it has been found in *Gryllus bimaculatus* (field cricket) that they can maintain circadian rhythms without input from CCN (Ohguro,

Moriyama and Tomioka, 2020), meaning the optic peripheral clock can regulate its own circadian light sensitivity pathways that otherwise inform such (Damulewicz et al., 2017). Similar can be found in plants where response levels to input signals are rhythmic with circadian regulation of PHYs and CRYs in plants being shown, demonstrating that circadian rhythms can determine plant clock light input pathway sensitivity (Toth et al., 2001). In mammals, based on *M. musculus* studies, the location of CCN appears to be the Suprachiasmatic nucleus (SCN) in the hypothalamus of the brain (Welsh, Takahashi and Kay, 2010). It has been recently found that retinal ganglion cells are the key neurons for determining CCN entrainment to light, specialised for low and long-term light sensitivity, in addition to supporting visual information from other retinal cells (Do and Yau, 2010).

In addition to light, temperature can often entrain CCN, though circadian clocks show temperature compensation in expressed rhythms, accounting for increased cellular activity at high temperatures (Pittendrigh, 1960, Sorek and Levy, 2012). In the absence of light, temperature can be a potent zeitgeber synchronizing CCN, shown in the Hemipteran *Pyrrhocoris apterus* (Firebug) (Kaniewska et al., 2020), with *D. melanogaster* studies showing additional neurons are harnessed to assist the maintenance of morning and evening activity peaks, especially when other CCN are absent or non-functional (Busza, Murad and Emery, 2007). Temperature has a similar accessory role to light entrainment across varying domains of life, likely due to its typical coupling with day/night cycles, though can become dominant depending on species-specific adaptions or relative rhythmic amplitudes (Rensing and Ruoff, 2002). Mammals too show that temperature is mostly a weaker zeitgeber to light, with *M. musculus* activity studies showing fewer and weaker rhythms in temperature only entrained individuals (Refinetti, 2010). However, temperature cycles can be significantly correlated with pest species behaviour, shown with *Solenopsis Invicta* (Red imported fire ant) (Lei et al., 2021) and can be a dominant zeitgeber in mammals, like shown for the first time in *Georychus capensis* (Cape mole-rat) and

Cryptomys hottentotus mahali (Mahali mole-rat) (Hart et al., 2021). These findings demonstrate that non-light zeitgebers can't be disregarded when considering species circadian clock entertainment to natural environments fluctuating in multiple zeitgebers at once. Constant light (L/L) can have the effect of running down circadian clock activity. Shown in both *M. musculus* and *D. melanogaster*, L/L can both directly rundown the cycling of the PER associated complexes and interfere with CCN signalling, acting on photoreceptive dCRY in *D. melanogaster* and through SCN neuron pathways in mammals (Marrus, Zeng and Rosbash, 1996, Emery et al., 2000, Chen et al., 2008, Helfrich-Forster, 2020). However in plants, circadian rhythms have been shown to be more robust under L/L conditions while becoming rapidly arrhythmic in constant dark (D/D) (Oakenfull and Davis, 2017). Research has shown however that CHS (Chalcone synthase):LUC (Luciferase) signals damped in rhythms over time in L/L with CAB (Chlorophyll a/b-binding protein):LUC signals remaining rhythmic for ~4+ days in L/L (CAB being important for chlorophyll activity while CHS for protection from sunlight induced damage) (Thain et al., 2002), maintaining period length of ~24.5h (Millar et al., 1995). Root tissue-specific clocks maintain robust rhythms into L/L also (Thain et al., 2002)

Feeding and signals from the gut can also have strong influences over circadian clock entrainment. In SCN ablated *M. musculus*, feeding rhythms were shown to be important entrainment signals, generating rhythmic activity (Sheward et al., 2007). Independent of CCN entrainment and also any light cycle, feeding can also entrain and phase shift peripheral clocks as shown in *Rattus norvegicus* (Common rat) liver cells (Stephan, Swann and Sisk, 1979, Stokkan et al., 2001). Some research suggests that across mammals feeding may be a more dominant factor controlling physiological rhythms (Hazlerigg and Tyler, 2019). However, there is little research on the effects of feeding, and at specific times of day, on insect entrainment of the CCN, though specific food types has been shown to be important at determining whether feeding rhythms are present (Niepoth et al., 2018).

1.2.2.2 Activity and avoidance

Perhaps the most studied and easiest to observe effects of the circadian clock is the circadian regulation of species activity rhythms, with evidence of numerous behaviours being expressed rhythmically and under constant conditions. The first reported observations of such came from plants with a legume, *Mimosa pudica* (Shameplant), maintaining rhythmic opening and closing of leaves in the absence of light (d'Ortous, 1729). However, it took time for the possibility of other zeitgebers informing such rhythms to be excluded and so prove true circadian rhythmicity in such behaviours (Somers, 1999). As mostly sessile organisms, plants rely on the precise timing of activities to best match the fluctuating environments around them due to a lack of mobility, leading to almost all plant physiology interacting with circadian regulation, providing significantly increased fitness (Michael et al., 2003, Michael and McClung, 2003, Dodd et al., 2005). Perhaps the most important aspect of plant physiology that relies on circadian cycles is photosynthesis, where it's been shown that *A. thaliana* relies on a functioning circadian clock to maximise efficiency and carbon fixation, significantly increasing photosynthetic productivity, likely predominantly through chlorophyll regulation (Dodd et al., 2005). Other pathways by which plants gain nutrients can also be under circadian regulation. *A. thaliana* research on water provision, which is also crucial for photosynthetic processes, demonstrated circadian regulation in response genes with time-dependent sensitivities and anticipation of increased drought conditions (Wilkins, Brautigam and Campbell, 2010). Temperature responses seem to be closely tied with that of drought (Liu et al., 1998) with plants also showing numerous time-dependent gene regulation changes after cold treatments (Bieniawska et al., 2008). Nutrients such as iron also showed interaction with the circadian clock where reducing iron content altered circadian phases with *cca1-lhy* clock mutants lacking such changes, however, not all nutrients have been found to interact with the plant clock (Hong et al., 2012). Nutrients gained from animals, like seen in insectivorous plants, likely occurs

rhythmically due to the activity rhythms seen in insects, yet so far no research has demonstrated how insectivorous plants may anticipate this using circadian rhythms, however, electrical signals similar to those used for *Dionaea muscipula* (Venus flytrap) trap closing may interact with such (Volkov, Adesina and Jovanov, 2007, Xie et al., 2021).

Animals have been shown across extensive amounts of research to show clear activity rhythms with precise timing of peaks in activity and troughs, often coupled with increased levels of sleep. *D. melanogaster* activity has been shown to have a crepuscular rhythm, showing activity around dawn and dusk. This behaviour shows prominent anticipation, increasing in activity before changes in light occur. The dusk activity bout has also been shown to be more robust with its amplitude remaining high during constant conditions as well as in clock neuron mutants that loose dawn activity peaks, specifically after the loss of crucial neuropeptides like PDF (pigment dispersing factors) (Hamblen-Coyle et al., 1992, Renn et al., 1999). PDF otherwise regulates photoperiodic responses in multiple recorded species (Yoshii et al., 2009, Shafer and Yao, 2014, Kotwica-Rolinska et al., 2022). Across insects there is often the maintenance of an evening peak in activity, likely due to thermoregulatory effects and predator avoidance (Heinrich, 1993, Malmqvist et al., 2018), in mammals however the maintenance of similar activity rhythms across orders is potentially rarer (Refinetti, 2007). Through *M. musculus*, which is a clear nocturnally active species, studies have shown how the mammalian clock regulates timed activity with clock mutants or CCN ablated individuals loosing entrained rhythmic behaviours (Antoch et al., 1997, Tousson and Meissl, 2004, Nakamura, Takasu and Nakamura, 2016, Takhashi et al., 2018). Activity irrelevant to time of day can also be seen in certain primates, where genera of Lemuridae activity revolves around interactions with other species or conspecifics. This ‘cathemeral’ activity appears to still be under circadian control and may be predictable by adaptions in the eye (Tattersall, 2008, Eppley, Ganzhorn and Donati, 2015). However, even within a species, individuals may present differing activity rhythms,

affecting important timed activity such as migration in songbirds. These individual differences in circadian regulation can be expressed as chronotypes, where it has been found to vary by ~6h in humans to ~23h in domestic cats (*Felis catus*), the largest difference between individuals within a species so far recorded (Refinetti et al., 2016, Rittenhouse, Robart and Watts, 2019). Studies on another mammal, *Meriones unguiculatus* (Mongolian gerbil), has shown distinct diurnal and nocturnal activity occurring at the same time within same species where ~1/4 are nocturnal irrespective of sex (Refinetti, 2006). The study and consideration of chronotypes within species may be important to analyse, however also may have minimal effect in natural environments with many fluctuating and interacting zeitgebers. Similar dissimilarities can also be seen in insects were there can be distinctions between male and female activity and their regulation of such, with *Nasonia* wasp females showing lack of activity rhythms in constant conditions compared to robustly rhythmic males with the same seen in *Plodia interpunctella* (Indianmeal moth) (Zavodska et al., 2012, Bertossa et al., 2013). Rhythms in activity can be very weak however with many *Oryctolagus cuniculus* (European rabbit) showing arrhythmic activity, though this doesn't exclude other aspects of their physiology being highly rhythmic, as is the case with *O. cuniculus* nursing rhythms and liver function (Jilge, 1993, Kennedy, Hudson and Armstrong, 1994, Piccione, Caola and Refinetti, 2007).

Sleep is also highly regulated, usually via homeostatic effects. The timing of sleep across the day however can show strong circadian rhythmicity with interactions with the circadian clock (Huber et al., 2004, Allada, Cirelli and Sehgal, 2017, Deboer, 2018). Sleep timing can be used as a way to avoid unfavourable conditions as well as modulate interactions with other species such as predators or prey, or simply reducing energy expenditure (Allison and Cicchetti, 1976, Roth, Rattenborg and Pravosudov, 2010). CCN transcription factors in mammals have been shown to be important for the regulation of sleep, with there being evidence of direct interactions between sleep homeostasis pathways and CCN from

sleep deprivation experiments and mutant studies (Achermann, 1999, Franken et al., 2000, Naylor et al., 2000, Deboer, 2018). Other forms of more direct inactivity or avoidance may also be under circadian regulation. At varying points across the day avoidance rates of environmental conditions can fluctuate, being shown to be under CCN control in respects to light in *D. melanogaster* (Baik et al., 2018). Avoidance of negatively associated environmental conditions has also been shown in *R. norvegicus* to display circadian rhythms that take time to re-entrain after phase shifts, demonstrating circadian control of these behaviours (Davies, Navaratnam and Redfern, 1973, 1974). Further to this, research has shown that both fear conditioning/learning, like that used to generate passive avoidance responses used in the aforementioned study, and aggression responses interact significantly with circadian rhythms (Lonowski, Levitt and Dickinson, 1975, Albrecht and Stork, 2017). Research into memory formation has also so far shown clear interactions with circadian clocks across both insects and mammals, with highly conserved molecular components, providing evidence similar interactions likely appear across most animals (Lyons and Roman, 2009, Gerstner and Yin, 2010). Attraction much like aversion may similarly be under circadian regulation, with thermotaxis shown in *D. melanogaster* and gravitaxis in *Euglena gracilis* (a Green aglae species) showing interactions with the circadian clock (Lebert, Porst and Hader, 1999, Bellemer, 2015).

1.2.2.3 Reproduction and development

Plant-pollinator interactions maintain high pollination efficiency, which can be provided by the entrainment of plant and pollinator circadian clocks (Bloch et al., 2017, Fenske et al., 2018). Plants attract pollinators through the emission of volatiles that act as either general or specialised attractants to species and the types of volatiles released often defines active pollinator communities (Burkle and Runyon, 2019). These volatiles are often released rhythmically with evidence of circadian control of such shown in *Petunia hybrida* (Garden petunia) where *lhy* was identified to control volatile emissions (Fenske et al.,

2015). Movement of flowers has also shown to be under circadian regulation, as seen in *Helianthus annuus* (Common sunflower) where time dependent movements significantly improve both growth and pollination efficiency (Atamian et al., 2016). Some flowering plants, such as *Silene colorata* (a species of Carnation), can specialize rhythms of volatile production and reward offerings to restrict visitation by a subset of pollinators and preferentially attract others, while retaining the plasticity to shift rhythms when pollinator interactions change. This demonstrates plant's ability to specialize to and interact with circadian rhythms of beneficial pollinators (Prieto-Benitez, Dotterl and Gimenez-Benavides, 2016, Lau et al., 2017). The circadian clocks of pollinators therefore have a major part in the dynamics of plant-pollinator networks. Pollinators show circadian rhythms in flower visitation that can be tightly correlated with plant pollen cycles (Gimenes, Benedito-Silva and Marques, 1996, Bloch et al., 2017) and there is evidence that memory involved in the navigation of plant-pollinator networks interacts with the circadian clock (Lehmann, Gustav and Galizia, 2011). The circadian clock is therefore a key aspect determining these networks and when such are OP with each other attraction and visitation is reduced, lowering pollination efficiency and therefore reducing fitness of both species involved. The interaction between plant-pollinator clocks are likely able to affect ecosystem communities from relatively few species, however making these systems more vulnerable to anthropogenic change that may disturb circadian rhythms suchas through pesticides or artificial light at night (ALAN) (Yon et al., 2016, Bloch et al., 2017, Fenske et al., 2018). Circadian regulation has been shown to play an important role in plant development with growth rate shown to occur rhythmically in plants, continuing into L/L conditions but significantly altered in *A. thaliana* core circadian oscillator mutants (Dawson-Day and Millar, 2002). Growth of florets and flowering structures in plants show clear interactions with the circadian clock, being shown to be specifically regulated by circadian oscillating cells in plant vasculature (Endo, Araki and Nagatani, 2016, Shim,

Kubota and Imaizumi, 2017). This demonstrates the importance of tissue-specific circadian clocks in plants that may not be local to affected tissues (Endo et al., 2014, Shimizu et al., 2015). Seed germination also shows circadian control with significant effects on gene expression depending on time of end of dormancy in seedlings, also demonstrating synced circadian responses across different clusters of seedlings (Zhong et al., 1998). Ending dormancy may be circadian regulated itself, providing fitness benefits (Baskin and Baskin, 1976, Penfield and Hall, 2009). Seed dispersal via animals, an important step of many plants' reproductive cycles, has been shown to benefit from circadian timing also (Guzman and Stevenson, 2011), but little research has shown how plants may attract or interact with frugivores and their circadian clocks.

Animals across many studies have shown precisely timed reproductive behaviours. Between male and female *R. norvegicus* circadian behaviours can be differentially expressed and change over time, highlighting differences between sex-linked behavioural needs (Krizo and Mintz, 2014), this may too include responses to reproductive behaviours and the circadian mechanisms controlling such. Findings also show circadian clock participation in the regulation of hormones. The precise timing of such in mammals shows importance for the maintenance of fertility levels and pregnancy (Karman and Tischkau, 2006, Sen and Hoffmann, 2020). However, the circadian regulation of sex pheromone release in mammals has had comparatively little research. The core circadian oscillator genes *Clk* and *Bmal1* have been shown to significantly affect fertility, demonstrating the importance of the circadian clock for optimal productive performance (Mereness, 2016, Zhang et al., 2016, Wang et al., 2017, Pan et al., 2020 Sen and Hoffmann, 2020). Research on clock mutant *M. musculus* also shows *Clk*'s importance at maintaining reproductive fertility (Kennaway, Voultsios and Boden, 2004). However, though these core clock genes appear to be highly important for reproductive maintenance, there are multiple redundant pathways that may help to regulate reproductive activity (Boden and Kennaway, 2006).

Testes are also one of the few mammalian organs found to lack circadian rhythmicity, yet the development of testes may interact with circadian clocks with reproductive maturity timing determined by circadian entrainment (Reiter, 1980, Foster, Ebling and Claypool, 1988, Morse et al., 2003). *M. musculus* developmental studies show circadian disruptions having significant effects on both physical and behavioural outcomes of pups (Smarr et al., 2017). Throughout development, mismatched timing can cause physiological issues and pathologies (Ebisuya and Briscoe, 2018), demonstrating the importance of precise timing that can be reliant on circadian clocks.

Circadian timing of insect development occurs at egg hatching, with rhythmic hatching of *Pectinophora gossypiella* (Pink bollworm) showing synchronisation by light pulses and maintenance into D/D conditions (Minis and Pittendrigh, 1968), with the same shown in *D. melanogaster* (Yadav, Thandapani and Sharma, 2014). Similar results have been seen in *Mansonia titillans* (a mosquito species) and *Triatoma infestans* (Kissing bug), however showing varying points of development where entrainment occurs (Nayar, Samarawickrema and Sauerman, 1973, Lazzari, 1991). Insect eggs can therefore maintain endogenous circadian rhythms with circadian clock genes identified in such. *per* mutant *Antheraea pernyi* (a silk moth) and *Bombyx mori* (Domestic silk moth) lose rhythmic hatching activity (Sauman et al., 1996, Ikeda et al., 2019) and *tim* mutant *B. mori* show significantly altered rhythms, demonstrating both core circadian oscillator genes are important for maintaining hatching rhythms (Nartey et al., 2020). Both temperature and light appear to have control over the entrainment of oviposition in *D. melanogaster*, with both factors having complex interactions with each other to produce bimodal rhythms (Kannan et al., 2012). Temperature and light also affect *Mamestra configurata* (Bertha armyworm), with egg development delayed at high and low temperatures, gating the initiation of reproductive calls, and calls themselves being significantly altered by changes in light (Geber and Howlader, 1986). Insect sex pheromones that act as attractants between

male and female conspecifics are also produced and released rhythmically which can be maintained into constant conditions (Hammack and Burkholder, 1976, Delisle and McNeil, 1987, Rosen, 2002). The specific responses to the presence of these volatiles are also mediated by circadian regulation with time-dependant responses to the presence of sex pheromones, shown in *Agrotis segetum* (Turnip moth) experiments to originate from CCN (Rosen, Han and Lofstedt, 2003). *Spodoptera littoralis* (African cotton leafworm) also shows circadian rhythmicity in its responses to sex pheromones, maintained to a certain extent into constant conditions. Increases in pheromone response are correlated with mating and locomotor behaviours, with CCN of *S. littoralis* potentially being entrained by provision of timed pheromone releases alone (Silvegren, Lofstedt and Rosen, 2005). However, another study showed that unlike *A. segetum*, *S. littoralis* pheromone response may be at least partially regulated by an antennal peripheral circadian clock, showing circadian rhythmic expression of clock genes and a pheromone degradation gene, potentially controlling maintenance of time-dependant pheromone responses (Merlin et al., 2007). When male and female *S. littoralis* were shifted OP with each other they had reduced reproductive efficiency, demonstrating how the matching of sex pheromone release and response rhythms has as functional effect on species fitness (Silvegren, Lofstedt and Rosen, 2005). Clock mutant *D. melanogaster* has significantly reduced fertility also, with males lacking a functional clock showing a significant decline in sperm (Beaver et al., 2002). Similar can be seen in *S. littoralis* where the release of sperm shows circadian rhythms continuing into D/D, demonstrating circadian control over sperm release (Bebas, Cymborowski and Giebultowicz, 2001). In *Lymantria dispar* (Gypsy moth), the disruption of circadian rhythms has severe effects on reproductive fitness, previously shown using L/L conditions which partially sterilised *L. dispar* (Giebultowicz et al., 1989).

1.2.2.4 Feeding and the gut

Timing of mammalian feeding varies across species but has been shown to likely be under circadian regulation, with circadian clock ablated *R. norvegicus* loosing rhythmic feeding patterns (Nagai et al., 1978) and many hormones involved in such being recorded to have circadian rhythms (Fleur et al., 1999, Ruiter et al., 2003, Bodosi et al., 2004). There are also consistent rhythms in features of the rumen and feeding in grazing mammals (Dove et al., 2007, Gregorini et al., 2018). Feeding itself is shown to be a strong zeitgeber for both CCN and peripheral circadian clocks, including the type of food affecting the regulation of numerous clock genes (Hazlerigg and Tyler, 2019, Pickel and Sung, 2020). However, research found CCN cannot be predominantly entrained by these peripheral clock signals and that CCN activity can hamper peripheral clock entrainment to food zeitgebers (Damiola et al., 2000, Ming et al., 2001).

Insect feeding can also occur rhythmically, with research on *Hadenoecus subterraneus* (Common cave cricket) showing maintenance of feeding behaviour rhythms in D/D (Reichle, Palmer and Park, 1965). Feeding rhythms shown in *S. littoralis* were demonstrated to persist into D/D conditions, with rhythmic expression of clock genes present in the gut (Suszczynska et al., 2017). Similar has been shown in blood sucking species, timing their activity with the presence of food sources, feeding rhythmically, shown in *Culex pipiens* (Common house mosquito) and *Lutzomyia longipalpis* (a sandfly species complex) with the latter also found to possess rhythmic circadian clock genes (Lazzari, Minoli and Barrozo, 2004 Meireles-Filho et al., 2006, Barrozo et al., 2010). Processes in the gut can also be influenced by circadian genes, where gut purging before *Samia Cynthia* (another silk moth species) undergoes pupation occurs rhythmically (Mizoguchi and Ishizaki, 1982) and signals from the presence of blood in the gut of *L. longipalpis* may interact or entrain circadian rhythms (Meireles-Filho et al., 2006). However, more research on the ability for food to entrain insect clocks, like carried out in

mammals, may be useful. *D. melanogaster* gut tissues maintain specific peripheral circadian clocks, similar to mammals, with the loss of gut peripheral clocks causing shifts in physiology (Xu, Zheng and Sehgal, 2008). These peripheral clocks may be informed by food and the type of such, potentially regulating further physiological circadian rhythms. Insect fat bodies and midgut maintaining circadian rhythms may be useful in detoxification of plant secondary metabolites where digestion and detoxification genes are rhythmically expressed to best suit the build up of plant material in the gut, shown in *Spodoptera litura* (tobacco cutworm) (Zhang et al., 2021).

Plants gain nutrients from the soil through root systems which show tissue specific circadian clocks which regulate the uptake of nutrients such as nitrogen. Root clocks have also been shown to have shorter periods than others in *M. trunctula* (Wang et al., 2021) while in *A. thaliana* root clocks have longer by comparison (James et al., 2008). Plants can also differentially express defence mechanisms to increase fitness (Michael and McClung, 2003, Dodd et al., 2005).

1.2.2.5 Immune system and detoxification

Plants have two distinct defensive pathways, one regulated by salicylic acid (SA) and one via jasmonic acid (JA). The circadian clock has been shown to have extensive regulatory control of both such pathways, in addition, the circadian control of such often leads to these two systems being in opposing phases to each other. SA mediated defences are often upregulated at night to best suit threats from microbial pathogens, while up-regulation of JA herbivory defence pathways occurs during the day when insect and other animal herbivores are most active. The precise timings of these two systems leads to increased plant fitness (Leon-Reyes et al., 2010, Pieterse et al., 2012, Goodspeed et al., 2013a, Tamaoki et al., 2013, Vos, Pieterse and Wees, 2013, Zhang, Bo and Wang, 2019). Plants also show clock regulated releases of metabolites that maintain beneficial soil

microbiomes, showing reduced diversity in the rhizosphere of clock mutant *A. thaliana* (Hubbard et al., 2018). Secondary metabolites from root tissues may also be under circadian regulation with the accumulation of nicotine in *Nicotiana attenuate* (Coyote tobacco) lost in clock mutants (Li et al., 2018) along with the regulation of JA and SA secondary metabolite pathways, with many herbivory defence mediated pathways interacting with the plant circadian clock (Joo et al., 2018, Zhang, Bo and Wang, 2019, Valim et al., 2020). Pathogen resistance shows circadian rhythmicity with maintenance into L/L which is lost in clock mutants (Bhardwaj et al., 2011). Similar is seen with herbivory defence via JA pathways, such as with the control of glucosinolate accumulation (Goodspeed et al., 2013a, Goodspeed et al., 2013b, Burow and Halkier, 2017, Lei et al., 2019). Detoxification can also occur rhythmically with circadian clock genes found to control the management of reactive oxygen species, shown to be maintained without the need for oxidative stress signals in *A. thaliana* (Lai et al., 2012). Plants have numerous detoxification pathways allowing them to manage the intrusion of toxic compounds from the environment, such as heavy metals. This has led to the study of phytoremediation where plants are used to decontaminate or improve soil quality (Coleman, Blake-Kalff and Davies, 1997, Widdup et al., 2015, Kumar et al., 2016, Buono et al., 2020). It's likely these pathways interact with plant circadian clocks however the application of such to understand and improve phytoremediation hasn't been widely researched.

The mammalian immune system has been extensively researched, due to its relation to human health and disease prevention, and it has been shown to be precisely regulated by circadian clocks (Labrecque and Cermakian, 2015, Poole and Kitchen, 2022). Research using animal models, such as *M. musculus*, have shown timed immune responses across the day, with certain pathways up and down regulated over time to best face appropriate potential threats, similar to such shown in plants. The circadian regulation of pathogen resistance also occurs, shown with *Diplococcus pneumoniae* (Streptococcus-related

bacterium) infections causing varying responses in *M. musculus* that changes in light insensitive individuals (Shackelford and Feigin, 1973). Similar responses to *Salmonella typhimurium* (Salmonella-related bacterium) infection were shown where inflammation responses show circadian rhythms and regulation, further highlighting the need for chronotherapeutic considerations in treatments (Bellet et al., 2013). Components of the innate immune system also show circadian rhythms with leukocytes changing in concentration and response over time (Brown and Dougherty, 1956, Scheiermann et al., 2012). Macrophages show similar regulation as well as specialised circadian clock gene expression (Keller et al., 2009, Collins et al., 2021). Circadian rhythms also interact with the gut microbiome which may have wide reaching effects on the health of the host and digestion related pathways (Frazier and Chang, 2020, Zhao et al., 2021). Detoxification rhythms under circadian regulation are also shown, with numerous studies demonstrating the liver peripheral circadian clock having a prominent role in controlling such. The loss of clock function in hepatic cells significantly altered detoxification pathways and increased overall susceptibility to certain products (Levi and Schibler, 2007, Zhao et al., 2019). Loss of central circadian clock genes recreate this effect, disrupting detoxification rhythms (Lin et al., 2019). Detoxification pathway rhythms can also be affected by exposure to the products they may act on, creating a complex interaction network that research is attempting to model to help improve the viability of chronotherapeutics (Miller et al., 1999, Currie et al., 2005, Giebultowicz, 2018).

Research on insect immune systems have identified digestion related organs as crucial for the mediation of pathogens and toxins, similar to the liver in mammals. The *D. melanogaster* fat body regulates immune responses and releases into haemolymph and has been shown to do so rhythmically, with food related entrainment of a peripheral clock, similar to mammals (Lemaitre and Hoffmann, 2007, Xu, et al., 2011). The level of research into the circadian rhythms of insect immune response factors is notably lower than in

mammals, however, research has shown that circadian clock regulated genes such as *Achilles* can regulate *D. melanogaster* immune responses (Li et al., 2017). Functional circadian clocks are required for maintenance of insect immunity and rhythmic resistance levels to specific pathogens such as *Pseudomonas aeruginosa*, with *per* and *tim* mutant *D. melanogaster* being shown to be immunocompromised (Shirasu-Hiza et al., 2007, Lee and Edery, 2008). An important factor of insect immune system responses has been found to be the priming of the innate immune system, which takes the place of an adaptive immune system seen in mammals (Pham et al., 2007, Kingsolver and Hardy, 2012, Cooper and Eleftherianos, 2017, Sheehan, Farrell and Kavanagh, 2020). However, the circadian regulation of such has so far not been shown, which may be important in determining the effectiveness of priming at specific times of day and therefore insect resistances to specific pathogenic attack. Circadian regulation of detoxification pathways involved with oxidative stress and damage mediation pathways show circadian rhythms, such rhythms being lost in *D. melanogaster per* mutants, which also have overall increased susceptibility (Krishnan, Davis and Giebultowicz, 2008, Beaver et al., 2012). Detoxification of plant secondary metabolites can also aid non-herbivorous species, where *Diadegma semiclausum* (Ichneumonid wasp species), an important natural enemy of the invasive pest *Plutella xylostella* (The Diamondback Moth), relies on its hosts detoxification pathways for optimal fitness (Sun et al., 2020). Therefore, interactions between rhythmic detoxification pathways and parasitoids are likely in other similar host-parasitoid/parasite relationships. The circadian control of detoxification of xenobiotics, a catch all term for foreign compounds within the body, has been shown through *D. melanogaster* studies, where Cytochrome P450s and other factors involved in innate responses to metabolize xenobiotics is under circadian clock control, with loss of rhythmic expression in *Clk* and *cyc* mutants (Wijnen and Young, 2006, Beaver et al., 2010). The role circadian clocks play

in the modulation of detoxification pathways can and has been implicated in the effectiveness of pest control treatments.

1.2.2.6 Plant defences, focusing on Brassicaceae

As discussed previously, plants have two distinct defence pathways adapted to either pathogenic or herbivorous attack. Pathways involving JA act as principal herbivory deterrents, ranging from development of phenotypes providing greater defence or regulation of secondary metabolites used against herbivores (Ruan et al., 2019). The biosynthesis of JA and how it interacts with the key signalling protein COI1 (Coronatine-insensitive 1) is shown in Fig. 1.3.

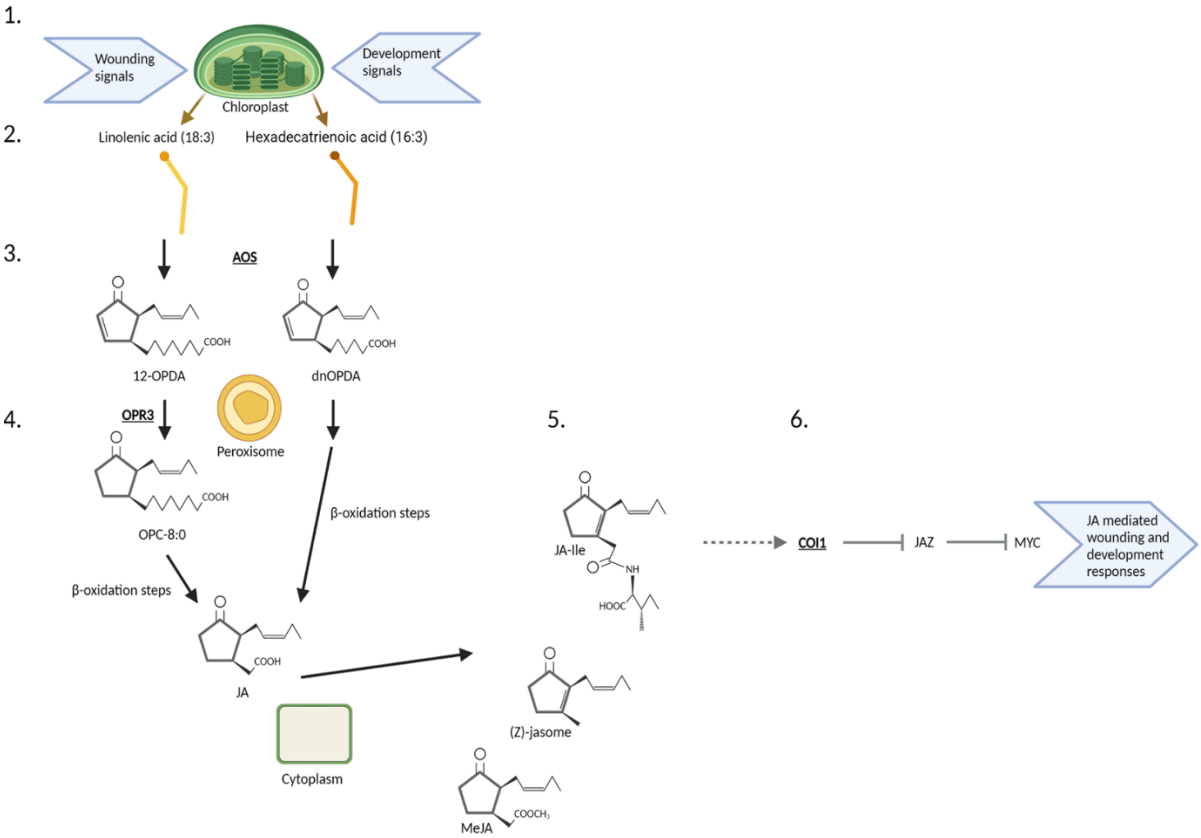


Figure 1.3 JA biosynthesis pathway and reception

Black arrows show metabolic production pathways, grey arrows show regulation with dotted arrow showing binding (activation) and flat head arrows indicating inhibition.

1. JA biosynthesis is regulated by both defence and developmental pathways e.g. systemin or PEP1 (Plant elicitor peptide 1) induced by mechanical damage (Li et al.,

2003) and DAD1 (Defender against cell death 1) involved in development (Ishiguro et al., 2001).

2. Fatty acids linolenic acid and hexadecatrienoic acid are released from phospholipid membranes in the chloroplast (Weber, Vick and Farmer, 1997, Wasternack and Hause, 2013, Chini et al., 2018).

3. These fatty acids are then converted to 12-OPDA (12-Oxo phytodienoic acid) and dn-OPDA (Dinor-Oxo phytodienoic acid) respectively via AOS (Allene oxide synthase) regulated pathways that likely controls both wound and developmental response pathways (Weber, Vick and Farmer, 1997, Kubigsteltig, Laudert and Weiler, 1999, Laudert and Weiler, 2002).

4. OPR3 (Oxophytodienoate-reductase 3) is then involved in the reduction of 12-OPDA to OPC-8:0 (3-oxo-2(2'-[Z]-pentenyl)cyclopentane-1-octanoic acid), leading to a number of β-oxidation steps that forms JA in the peroxisome (Schaller et al., 2000, Stintzi and Browse, 2000). It has also been shown that the loss of OPR3 for this step reduces male fertility (Stintzi and Browse, 2000) and responses to insect herbivory (Stintzi et al., 2001). However, dn-OPDA has been shown to undergo a OPR3 independent pathway leading to the production of JA precursor molecules in *opr3* mutants (Chini et al., 2018, Wasternack and Hause, 2018).

5. JA can then be further diversified in the cytoplasm, such as through further β-oxidation steps with (Z)-jasmone, an important secondary metabolite (Birkett et al., 2000) along with JA-Ile (Jasmonoyl isoleucine) via JAR1 (Jarin-1) (Schuman et al., 2018). JA can also be methylated to form MeJA (Methyl jasmonate), a crucial regulator of plant defence and development (Seo et al., 2001).

6. COI1 acts as a key JA sensitive signalling protein that regulates JA action on multiple downstream pathways, this being shown through the inhibition of JAZ (Jasmonate-zim domain) proteins after activation by JA-Ile, which then represses MYC (Xie et al., 1998, Chini et al., 2007, Sheard et al., 2010). This pathway is key to the regulation of JA regulated responses with mutants in such also showing increased herbivory damage (Chini et al., 2007, Fernandez-Calvo et al., 2011). However certain intermediary products of the JA biosynthesis pathways may induce responses independent of COI1 signalling (Blechert et al., 1997, Stintzi and Browse, 2000).

The circadian clock shows extensive control of these hormonal pathways, maintain precise rhythms of expression (Zhang, Bo and Wang, 2019). In addition to endogenous rhythms in the regulation of JA, JA may also be induced (Ruan et al., 2019). Wounding can trigger

damage-associated molecular patterns (DAMPs) that can upregulate the pathways shown in Fig. 1.3 (Hou et al., 2019). Systemins released after herbivory damage, such as that from caterpillars, activates JA pathways (Orozco-Cardenas, McGurl and Ryan, 1993, Schilmiller and Howe, 2005) and the accumulation of gene products crucial in these pathways can be time-dependent with potentially specific responses to insect herbivory compared to general damage (Reymond et al., 2000, Joo et al., 2019). The sensitivity of these responses and interactions between specific pest and plant host priming of herbivory defence can be time-dependent, with specific periods shown to induce increased secondary metabolite accumulation in brassicas with increased priming to herbivorous attack (Doghri et al., 2022). Similar can be seen in innate immune responses to pathogen-associated molecular patterns (PAMPs) (Sharma and Bhatt. 2015). It has been shown that the evening genes ELF4, 3 and LUX form an 'evening complex' and directly inhibit MYC genes (MYC2) shown in Fig. 1.3 (Zhang et al., 2018), therefore inhibiting JA pathways during dark conditions, demonstrating a key pathway by which herbivory deterrents such as glucosinolates are down regulated at night compared to the day (Thines, Parlan and Fulton, 2019).

The Brassicaceae plant family is categorised by their generally herbaceous forms in the *Brassica* genus, many of which used in agriculture, producing numerous farmed varieties from species such as *B. rapa* and *Brassica oleracea* (Wild cabbage) (Chen et al., 2011, Muimba-Kankolongo, 2018). This family is also known to produce glucosinolates which are the key component of a defensive pathway unique to the family, the Mustard Oil Bomb (MOB), producing an array of protective molecules, mainly isothiocyanates, which act as toxic herbivory deterrents (Fenwick and Heaney 1983, Singh, 2017, Wang et al., 2019). Recent studies have also showed potential health benefits for the consumption and increase in uptake of glucosinolates and their hydrolysis products in humans (Barba et al., 2016, Samec and Salopek-Sondi, 2019).

Glucosinolates are produced via JA pathways and categorised between aliphatic, indolic and benzenic biosynthesis pathways, determined by their starting amino acids used in their formation. Aliphatic glucosinolates derive from Ala, Leu, Ile, Val, and Met, Benzenic from Phe and Tyr, and Indolic from Trp. These differences affect the role, expression and response levels of the varying forms of glucosinolate (Sonderby, Geu-Flores and Halkier 2010, Agerbirk and Olsen, 2012). JA has a significant role in the expression of glucosinolates, potentially upregulating glucosinolate production in response to MAMPs and PAMPs (Brader, E and Palva, 2001, Kastell et al., 2013, Sánchez-Pujante et al., 2018).

There has also been significant research into the genes responsible for glucosinolate production (Sonderby, Geu-Flores and Halkier 2010), identifying a specific protein complex that may be used to increase glucosinolate content (Frerigmann, Hoecker and Gigolashvili, 2021). Mutants of genes involved at certain points of *A. thaliana* JA pathways, also a member of Brassicaceae, have been identified. *coi1-16*, a specific *coi* point mutation, generates JA insensitive plants that can still be maintained at lower temperatures (≤16°C) while significantly affecting jasmonate signalling (Ellis and Turner, 2002). Mutants have also been found that interrupt JA biosynthesis such as *aos* (Park et al., 2002) and *opr3-1* (Schaller et al., 2000, Stintzi and browse, 2000) mutants. The proteins these mutants affect are highlighted in Fig. 1.3. The catalogue of mutants provided by *A. thaliana* research allows for investigation into the effects defence signalling has on plant physiology and interactions with herbivores.

Plant myrosinase is released from tissue compartments upon physical damage, interacting with glucosinolate (Matile, 1980). Once in contact a complex forms and myrosinase cleaves a glucose molecule from glucosinolate, generating an unstable intermediary product. This breaks down into the active toxic products associated with the MOB, depending on certain abiotic factors such as temperature and pH (Ratzka et al., 2002, Kliebenstein, Kroymann and Mitchell-Olds, 2005, Kissen, Rossiter and Bones, 2009,

Hanschen et al., 2017). Plants enhance glucosinolate content within tissues in response to herbivore attack and wounding signals (Textore and Gershenzon, 2008), however glucosinolate levels can also be altered through plant endogenous factors such as glucose concentration (Guo et al., 2012) and variations in expression of certain genes (Burow et al., 2015) and protein interactions (Frerigmann, Hoecker and Gigolashvili, 2021). Glucosinolate has also been shown to be expressed rhythmically with time-dependent responses (Goodspeed et al., 2013b, Doghri et al., 2022). Loss of both the transcription factors MYB28 and MYB29 prevents the production of aliphatic glucosinolates, those likely to be most important for herbivory defences against leaf herbivores, compared to indolic glucosinolates that are still expressed. These changes in glucosinolate production and profiles significantly affects the outcome of herbivory and pest species entrainment (Beekwilder et al., 2004, Sonderby et al., 2007, Sonderby, Geu-Flores and Halkier 2010, Goodspeed et al., 2013b).

1.2.3 Chronotoxicity

Chronotoxicity is the study of species susceptibility to treatments and how they change over time, specifically according to species' endogenous circadian rhythms which may be useful for informing treatments such as those used in pest management. However, it is important to consider the factors involved in the regulation of time-dependent responses and what role circadian clocks may play in the maintenance of such and overall levels of resistance (Pszczolkowski, 2008).

1.2.3.1 Plants

The plant circadian clock shows extensive control, interacting with almost all aspects of plant physiology (Michael and McClung, 2003, Singh and Mas, 2018), therefore including responses to toxicity. Different plant tissues can have varying circadian clocks which act on specific processes (Davis, Endo and Locke, 2022), these clocks may also have differing

endogenous rhythms as shown between *A. thaliana* and *M. trunctula*, where the former's root clock has a longer period than shoot clocks and shorter in the latter (James et al., 2008, Wang et al., 2021). Therefore, identifying detoxification pathways and the specific circadian clocks controlling such is important, which is becoming more accessible with the use of tissue-specific analysis methods with high temporal resolution. Some of these methods take advantage of advanced genetic tools such as CRISPR-Cas9 (Clustered regularly interspaced short palindromic repeats and CRISPR-associated protein 9) (Decaestecker et al., 2019) or use more traditional techniques such as with thermal or luciferase imaging (Dakhiya and Green, 2019, Roman et al., 2019). Circadian clock genes have been found to regulate plant immune responses such as *xct* (*XAP5 circadian timekeeper*), which highlights how plant responses to toxin presence from pathogens can vary across the day (Xu et al., 2017). Responses to chemical treatments like glyphosate, a broad-spectrum herbicide and the most common active ingredient in such, has been shown to have circadian rhythms. The use of glyphosate at 6:00pm reduced weed biomass over time by ~5x compared to use at 6:00am, demonstrating a significantly different level of resistance depending on the time of day. A significant part of the differences found on one species was associated with rhythmic leaf movements, shown with *Abutilon theophrasti* (Velvetleaf, a mallow plant) (Mohr, Sellers and Smeda, 2017). Glyphosate acts on aromatic amino acids, blocking their biosynthesis via interference of the shikimate pathway (Steinrucken and Amrhein, 1980, Funke et al., 2006), however, it appears that part of these effects can be mediated via circadian clock pathways as shown in PHY mutant *A. thaliana*. Typically, the shikimate pathway shows circadian rhythms and evidence of circadian regulation determining glyphosate effective minimal concentration (Sharkhuu et al., 2014, Belbin et al., 2019). The mutant gene *gre1* (*glyphosate response 1*) generated glyphosate resistant *A. thaliana* via disrupting PHY B pathways which in high red light conditions, which PHYs are sensitive to, the genes involved in the shikimate

pathway are upregulated (Sharkhuu et al., 2014), leading to increased glyphosate resistance (Yang et al., 2017). Core circadian oscillator gene mutant *cca1-ox* (*circadian clock associated – overexpressor*) showed a loss in rhythmic glyphosate resistance, further demonstrating the circadian regulation of plant herbicide resistance (Belbin et al., 2019). Many other herbicides show rhythmic efficacies with a study comparing the rhythmic effectiveness of 4 different classes of herbicides, each with specific action pathways including glyphosate, finding rhythmic responses to each. In addition, it was also found that increasing the quantity of herbicides used did not overcome circadian rhythms in resistance levels, helping to demonstrate that considering timing of treatments is of economic and environmental importance (Miller et al., 2002). These finding also demonstrate that rhythmic resistance to treatments is likely widespread.

1.2.3.2 Mammals

As similarly shown in plants, mammals possess tissue specific peripheral clocks, often kept entrained by signals from CCN but are not dependent on it to function (Yoo et al., 2004). Many properties of the immune system are shown to be under circadian regulation with the abundance and activity of immune cells showing circadian rhythms including immune cell specific circadian clocks (Keller et al., 2009, Scheiermann, Kunisaki, Frenette, 2013, Golan et al., 2018). Responses to pathogens and their toxins was first shown to occur with circadian rhythmicity in the 1960's where *Escherichia coli* toxins were found to have varying severity of effects on *M. musculus* across the day. The immune system was also upregulated in time with the active phase, when *M. musculus* would most likely encounter xenobiotics and injury (Halberg et al., 1960). Repair and regeneration was demonstrated to be under circadian control also showing maintenance of circadian clocks in *M. musculus* fibroblasts, with research also showing how the recovery of tissues and cell division can be time-dependent (Matsuo et al., 2003, Nagoshi et al., 2004). In addition, this may too relate to tumour suppression and responses to radiation. *M. musculus per2* mutants show

significant losses in tumour suppression activity with loss of circadian rhythms. Radiation therefore had a more severe effect on *M. musculus per2* mutants, causing significantly increased tumour development compared to wild-type *M. musculus* that maintained circadian rhythmicity. In *M. musculus* that maintained rhythms, time-dependent responses to radiation exposure was found with 2:00am exposure in 7:00am-7:00pm day/night cycles causing the most severe effects (Pizzarello et al., 1964, Fu et al., 2002). The chronotoxicity of radiation and circadian rhythms may therefore be applicable to cancer treatment, where the disruption of circadian rhythms in cancer cells, though may be a step that leads to proliferation, may be harnessed to increase treatment efficacy (Sulli et al., 2018, Sulli, Lam and Panda, 2019, Alamoudi, 2021, Lee, 2021, Zhang et al., 2021). The liver peripheral clock was shown to be important for detoxification of xenobiotic compounds and can often be entrained by feeding rhythms, potentially as this constitutes the most likely pathway for xenobiotic entry (Stokkan et al., 2001). Genes involved in detoxification, such as cytochrome P450s, have been shown to be regulated by the circadian clock including genes such as *Clk* (Akhtar et al., 2002, Oishi et al., 2003), and when clock genes are knocked out, *M. musculus* becomes hypersensitive to xenobiotics (Gachon et al., 2006). Direct injections of bacterial toxins show significant time-dependent effects where *M. musculus* that had streptomycin injections during light phase survived longer and showed lower levels of toxicity compared to those injected during dark phase (Yoshioka et al., 2020). Time-dependent toxicity levels was also found for the histamine antagonist cetirizine in *M. musculus*, where mortality and body weight were significantly affected by time of oral dosing (Dridi, Mansour and Boughattas, 2010). Sodium valproate, often used to treat epilepsy in humans, was shown to display varying levels of chronotoxicity, causing the highest mortality at 5:00pm (3h prior to dark phase) in *M. musculus* while however not showing any difference in anti-convulsive effects, demonstrating toxicity and neuropathic effects can be under different circadian regulation (Ohdo, Nakano and Ogaway, 1988).

Chronotoxicity through neuropathic effects was however shown in *R. norvegicus*, where treatment with carbachol during dark phase showed significantly increased aggression and cohabitating *M. musculus* mortality compared to light phase treatments (Lonowski, Levitt and Dickinson, 1975). Chronotoxicity is also evident in human studies, though far fewer in number, however, oleander poisoning was shown to have significant time-dependent effects where ingestion during the evening had significantly improved outcomes (Carroll et al., 2012). Specific diets and time of feeding have also been shown to impact chronotoxicity, where low-sodium diets in *M. musculus* increased overall toxicity levels without altering rhythms (Shito et al., 1991) and time restricted feeding in another study significantly affected circadian rhythms and toxicity levels (Song et al., 1993). These findings demonstrate how differing factors can influence time-dependent toxicity levels.

1.2.3.3 Insects

Insect chronotoxicity has been studied since *Blattella germanica* (German coackroach) was treated with Potassium cyanide and showed clear rhythms in susceptibility in the 1960's (Beck, 1963). Since then *B. germanica* chronotoxicity continues to be studied, uncovering how the circadian regulation of glutathione-s-transferase (GST) is responsible for the modulation of an insecticide permethrin, becoming most resistant during the dark phase, with maintenance of rhythms into D/D, demonstrating endogenous circadian clock control (Lin et al., 2014). *D. melanogaster* was also used to show if GST and other detoxification genes displayed circadian rhythms, finding there was significant daily variation in such. Groups of detoxification genes were highly expressed after midday which is similar to when *D. melanogaster* tested showed the greatest resistance to propoxur and fipronil pesticides. However, propoxur resistance rhythms were lost in L/L conditions and other pesticides such as deltamethrin and malathion showed no rhythms in any conditions (Hooven et al., 2009). This demonstrates that not all pesticides act on pathways under circadian regulation. Specific tissues and peripheral clocks in insects may be more

responsible for detoxification similar to the liver in mammals. The fat body is the predominant store of energy in insects but also regulates numerous physiological functions including immunity and detoxification (Arrese and Soulages, 2010, Skowronek, Wojcik and Strachecka, 2021). Additionally, it has been shown to maintain a peripheral clock that can be entrained with or without the need of CCN, either through maintenance of peripheral clock rhythms or feeding (Xu, Zheng and Sehgal, 2008, Xu et al., 2011, Sehgal, 2016). The fat body has been shown to regulate metabolic pathways including the control of cytochrome P450s, also regulating the detoxification of molecules such as nitrogen (Keeley, 1985, Li et al., 2019). The midgut may play a significant role in the regulation of circadian rhythms interacting with feeding, with *A. pernyi* studies showing this is dependent on CCN (Sauman and Reppert, 1998). However, a study on *B. mori* showed that not only does the midgut maintain peripheral circadian clock rhythms but can do so without CCN or sensory organs, demonstrating light receptive pathways in the midgut (Nobata et al., 2012). These differing findings demonstrates possible species-specific variation in the maintenance and entrainment of peripheral clocks and thus interactions with detoxification pathways and chronotoxicity in insects. It has also been shown that disruption of circadian clock oscillator genes *per* and *tim* in *D. melanogaster* did not change resistance rhythms, but that resistance was controlled by *cyc* and *Clk* interacting with PDP1 (PAR-domain protein 1). PDP1 regulates detoxification pathways in *D. melanogaster*, which showed increased mortality in *cyc* and *Clk* mutants to permethrin and malathion pesticides (Beaver et al., 2010). In *S. litura*, detoxification genes were found to be interacting with circadian clock genes and demonstrated chronotoxicity to imidacloprid insecticide, with ~20% higher mortality when applied during the dark phase. This indicates that by feeding at night *S. litura* may avoid exposure to predators but also pesticide spraying when they might be most susceptible (Zhang et al., 2021). *Musca domestica* (Housefly) has also been used to demonstrate chronotoxicity to a number of pesticides

including Dichlorodiphenyltrichloroethane (DDT). For each pesticide used, *M. domestica* showed highest levels of susceptibility 1h before lights on, including after switches between 6:00am lights on and 6:00pm lights on entrainments. There was also a loss of rhythms in D/D conditions (Shipp and Otton et al., 1976). These findings demonstrated that *M. domestica* susceptibility to pesticides was likely not under circadian clock control, but, may be influenced by peak feeding, which occurs around the dawn activity peak in *D. melanogaster* (Lin et al., 2014). As previously shown, feeding may entrain gut related peripheral clocks involved in detoxification, leading to the aforementioned findings. The expression rhythms of GST in the fat body of *B. germanica* showed circadian clock control, but only appeared to influence toxicity resistance during the dark phase (Lin et al., 2014), demonstrating how different immune and detoxification pathways can be involved in the mediation of pesticide treatments at different points in time. These previously mentioned factors highlight how chronotoxicity is a complicated process with numerous interacting pathways. However, if appropriately harnessed, these factors also show that chronotoxicological considerations could significantly improve the efficacy of treatments, improving the economic and environmental impacts of pest management strategies.

1.2.4 Harnessing circadian rhythms for pest management strategies

With numerous cases of chronotoxicity found across species, including the extensive research demonstrating the wide-reaching control circadian clocks can have in an organism, there are many avenues of research that may allow for the control of species via human intervention.

1.2.4.1 Harnessing the central circadian system

The phase entrainment of circadian clocks for both CCN and peripheral tissue clocks has been shown to be important for the maintenance of resistance (Scheiermann, Kunisaki and Frenette, 2013, Goodspeed et al., 2013b), metabolic pathways (Pickel and Sung, 2020) and

behavioural interactions (Silvegren, Lofstedt and Rosen, 2005, Fenske et al., 2018), often loosing fitness if such becomes out of sync (Beaver et al., 2002, Dodd et al., 2005, Bloch et al., 2017, Haspel et al., 2020). Therefore, understanding what entrains circadian clocks and how their phases can be shifted may be of use in pest management.

It has been shown when plant circadian clocks are OP with that of a generalist pest, the pest has increased growth and the plant host increased leaf area loss (Goodspeed et al., 2013b). In addition, depending on the phase of plant circadian clocks, pollination efficiencies can be significantly altered (Fenske et al., 2018) along with susceptibility to herbicidal treatments (Sharkhuu et al., 2014, Belbin et al., 2019). The plant clock is most predominantly entrained by light schedules (Fankhauser and Staiger, 2002), but can be affected by temperature (Rensing and Ruoff, 2002) with tissue-specific clocks showing high levels of entrainment to cycles of species interactions (Frund, Dormann and Tscharntke, 2011). This includes interactions with specific molecules such as nitrogen (Gutierrez et al., 2008) and iron (Chen et al., 2013). These factors therefore provide a suite of possible exploitable plant clock entrainment pathways. Studying how genes are regulated in these pathways may open further avenues to control plant clocks and so control rhythms in growth, resistance and reproduction. Genes affected in these entrainment pathways can be used in genomics/transcriptomic studies, using circadian clock tissue-based assays to show daily variations in expression and how they relate to physiological features such as resistance (Panda et al., 2002, Covington et al., 2008). This may be used to identify pathways that provide resistance to pests through natural plant defences or specific secondary metabolites, in addition to susceptibilities to herbicides for removing weed species such as shown in *gre1* mutant *A. theophrasti* and circadian clock oscillator mutants in *A. thaliana* (Sharkhuu et al., 2014, Belbin et al., 2019).

Entrainment pathways in animals can also be considerably varied, with light entrainment through CCN clear in both insects and mammals (Roenneberg and Merrow, 2000, Schmal,

Herzel and Myung, 2020), however light is not necessarily a dominant zeitgeber over others such as temperature (Refinetti, 2010, Kannan et al., 2012) and feeding, particularly in peripheral clocks (Damiola et al., 2000, Schibler, Ripperger and Brown, 2003). Receptors on a *D. melanogaster* peripheral sensory organ (chordotonal organs) along with other circadian clock pathways have been shown to entrain circadian rhythms to temperature cycles with an amplitude of as little as 2°C (Sehadova et al., 2009, Wolfgang et al., 2013 Chen et al., 2015). Though the entrainment of the insect fat body through feeding has had relatively little research, similar functions of the mammalian liver circadian clock has been widely shown to be entrained by food intake, acting on oxyntomodulin shown in *M. musculus* to induce *Per1* and *Per2* expression in the liver peripheral circadian clock (Landgraf et al., 2015, Stokkan et al., 2001). The OP entrainment between body clocks leads to significant health effects, such as metabolic syndrome in mammals (Mukherji et al., 2015, Pickel and Sung, 2020). Phase entrainment in behaviour can also be pushed OP with endogenous rhythms where *Acomys russatus* (Golden spiny mouse) free-running rhythms are nocturnal, but in the wild, exposed to certain zeitgebers, becomes diurnal (Levy, Dayan and Kronfeld-Schor, 2006). Varying responses to light at night can also be seen between different species (Prugh and Golden, 2014, Owens and Lewis, 2018). Like discussed for plants, understanding the pathways that entrain the physiological rhythms of mammals and insects presents an important opportunity, to not only identify the genes responsible for the regulation and mediation of treatments, but also the potential manipulation of pest species by exploiting pathways to shift target species circadian rhythms.

1.2.4.2 Harnessing rhythms in the immune system

An aspect of biology that may be exploited are rhythms in immune system function. The benefits of knowing the specific daily rhythms in certain immune system pathways is

clearly shown with chronotherapeutics, with chronotoxicity in pathogen exposure also being shown.

Plant pathogens likely interact with SA regulated pathways, though its biosynthesis and therefore regulation can differ between plant species (Lefevere, Bauters and Gheysen, 2020), with base levels of both JA and SA shown to be under circadian regulation. JA accumulation may dictate resistance to generalist pest attack by *Trichoplusia ni* (Cabbage looper), with multiple studies showing evidence for the circadian control of JA, which may be regulated by ST2A (Sulfotransferase 2A) in *A. thaliana* (Goodspeed et al., 2013a, Yamashino, Kitayama and Mizuno, 2013). CHE (CCA1 hiking expedition) transcription factors were found to be activators for SA biosynthesis and interact with the circadian clock (Zheng et al., 2015). A phosphate transporter PHT4;1 (Phosphate transporter 4;1) was also found as a SA regulatory gene interacting with the circadian clock via CCA1 (Wang et al., 2014). Though specific regulatory pathways are currently unclear and may be complex, circadian regulation of such appears to be present between species and studies. This creates opportunities for chronotherapeutics/chronotoxicity to be applied to both unwanted and crop plants, creating treatment schedules to protect plants when they are most vulnerable or target the upregulation of immune pathways across the day (or opposite for weeds), including specific herbivore, pathogen and or toxin releases.

Insect and mammalian immune research has too found circadian control with multiple immune cell specific clocks. Insect innate immune systems vary in specific components abundance and responses across the day, demonstrating circadian rhythms that are lost in *per* and *tim* clock mutant *D. melanogaster*, suffering significantly increased mortality to both *Streptococcus pneumonia* and *Listeria monocytogenes* (Listeria) (Shirasu-Hiza et al., 2007, Lee and Edery, 2008, Li et al., 2017). Primming of the innate immune system could have varying sensitivity across the day, so may need to be considered along with daily resistance levels to any pathogenic treatments used against insect pests (Sheehan, Farrell

and Kavanagh, 2020). The mammalian innate immune system has multiple cells that maintain circadian clocks (Keller et al., 2009, Scheiermann et al., 2012), potentially demonstrating the importance of maintained circadian rhythms for the effectiveness of immune responses. Less research has shown immune cell circadian clocks in insects. Overall immune responses such as against *D. pneumoniae* and COVID-19 virus (Shackelford and Feigin, 1973, Liu, Ting and Zhuang, 2021), in addition to inflammatory, repair and radiation responses, all show circadian rhythms with evidence of circadian clock control (Pizzarello et al., 1964, Nagoshi et al., 2004, Bellet et al., 2013). Harnessing of such circadian rhythms has become prevalent in human medicine, with studies considering chronotherapeutics increasing with frequency over time since the 1990's, plotted in Fig. 1.4A. In addition, continued research may allow the modelling of circadian interaction networks to improve viability of chronotherapeutics (Miller et al., 1999, Currie et al., 2005, Giebultowicz, 2018). This highlights the success to which circadian rhythms can be applied to the improvement of treatments and outcomes, which may also be applied to the chronotoxicity of treatments against pests, which the study of has too increased over time though to a lesser extent, shown in Fig. 1.4B, bearing in mind the notable increase in overall scientific publications over time.

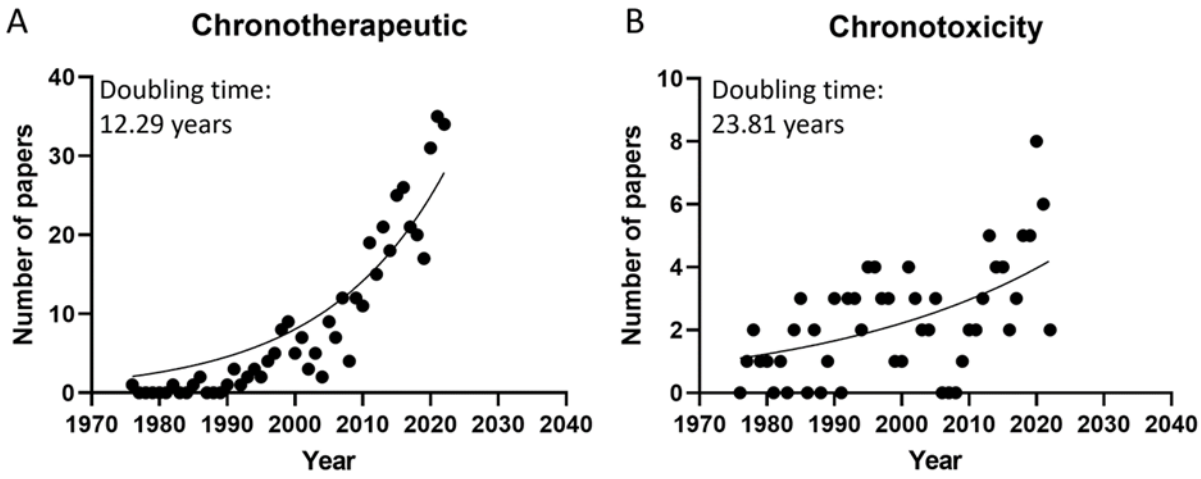


Figure 1.4 Appearance of key word A, chronotherapeutic, B, chronotoxicity in papers from search of Web of Science Core Collection all fields.

Key words must be used in either title or abstract. 2022 recorded up to June then doubled. Plotted using Graph Pad Prism 9 (GraphPad Software, 2020), using exponential growth equation to calculate doubling time.

1.2.4.3 Harnessing metabolism and feeding rhythms

The permeability of photosynthetic tissues and the intake of nutrients has been shown to occur rhythmically in plants (Zhang, Romheld and Marschner, 1991, Dodd et al., 2015, Hassidim et al., 2017). The variations in uptake across time and plant species (Haydon, Roman and Arshad, 2015) may lead to variations in chronotoxicity as shown in weeds such as *A. theophrasti* and in *A. thaliana* experiments (Mohr, Sellers and Smeda, 2017, Belbin et al., 2019). This presents easily exploitable rhythms where treatments at certain times of day will have greater uptake by plants, reducing the amount required, lowering the economic and environmental impacts.

Research on insects and mammals shows clear rhythmic feeding behaviours in numerous species, however, rhythmic profiles interact with species-specific pressures and adaptions leading to selection between variations of diurnal, nocturnal and crepuscular behaviours. Mammals display such feeding rhythms with *M. musculus* showing daily activity rhythms, feeding nocturnally, including during subjective (s) night after 10 days in L/L, though with altered rhythmic features (Possidente and Birnbaum, 1979). Daily feeding rhythms were also observed in *Capra hircus* (Domestic goat) breeds with time-dependent food choice (Rossi and Scharrer. 1992, Solanki, 1994). Predatory mammals such as *Vulpes vulpes* (Red fox) and *Lynx lynx* (Eurasian lynx) both maintained precise activity patterns, including as natural photoperiod changed over the year, demonstrating preferred hunting periods (Heurich et al., 2014, Kammerle, Rondeaux and Storch, 2020). When metabolism is shifted from entrained cycles there can be effects such as increased fat storage, increased disease incidences and pathologies and subsequently increased mortality rates (Mukherji et al., 2015, Pickel and Sung, 2020). Therefore, timing of feeding and regulation of metabolism

can be exploitable, potentially through the consumption of ingestible pest treatments and dictating exposure to such. These rhythms likely also inform how species breakdown treatments into either toxic or non-toxic by-products, affecting the overall efficacy of treatments. This can be applied to insect pests that show similar time-dependent feeding and metabolic function. *D. melanogaster* shows highest feeding rate around the dawn activity peak, also seen in *Drosophila suzukii* (Spotted wing drosophila), a closely related pest species (Lin et al., 2014). *S. littoralis* however shows nocturnal feeding rhythms (Suszczynska et al., 2017) and another closely related species *S. litura* feeds only during the dark phase, allowing it to avoid predation and most likely pest control treatments. *S. litura* digestive enzymes are upregulated in the light phase also, potentially further increasing detoxification (Zhang et al., 2021).

1.2.4.4 Harnessing rhythms in detoxification

Detoxification pathways most prominently relate to features of chronotoxicity and can determine the ability for species to process xenobiotics while mediating deleterious effects.

Circadian rhythms in plant detoxification pathways through cytochrome P450s and GST can be dependent on light cycles with a number of herbicides that target photosynthetic pathways also relying on such (Galle et al., 2019). This demonstrates the importance of considering the time of day in plants ability to mediate herbicidal treatments. GST responses are shown to be useful in mediating the effects of toxins such as arsenic (Kumar and Trivedi, 2018) and have been shown to have circadian rhythms in response to light in *Solanum lycopersicum* (Tomato) (Galle et al., 2018). These findings, in addition to the previous responses discussed, show that plants can be heavily affected by the precise timing of many different classes of treatments. They also show many identifiable pathways of defence and detoxification genes that could be exploited through regulation or silencing,

with the disruption of circadian genes themselves having significant negative effects on plant fitness (Dodd et al., 2005).

Detoxification pathways in insects and mammals show similar interactions with time-dependent expression of P450s and GST also shown, both of which have rhythms dependent on circadian clock genes which when lost causes significant increases in mortality and other negative outcomes (Wijnen and Young, 2006, Beaver et al., 2010, Lin et al., 2014). This, in addition to regulation of other detoxification pathways and therefore potentially treatment resistance, is seen across insects and mammals, with loss of rhythms and resistance often shown in clock mutants (Levi and Schibler, 2007, Krishnan, Davis and Giebultowicz, 2008, Beaver et al., 2012, Lin et al., 2019, Zhao et al., 2019). Understanding the timing of species detoxification will therefore be crucial for the enhancement of pest treatments efficacy, lowering costs and environmental damages as discussed with chronotoxicity.

1.2.4.5 Harnessing behavioural, reproduction and developmental rhythms

Specific behaviours are also affected by circadian rhythms, with timings of treatments causing significantly increased effects on such, potentially causing increased disruption to reproduction and development.

Plant leaf movements are a factor increasing plant susceptibility to herbicides, which has been shown to occur rhythmically under circadian clock control (Edwards and Millar 2007, Mohr, Sellers and Smeda, 2017). The specific timing of treatments affecting aggression and avoidance behaviours in mammals was also shown to have significant effect on outcomes (Lonowski, Levitt and Dickinson, 1975, Albrecht and Stork, 2017). Aggression in insects shows daily cycles, with increases in *Gryllus firmus* (Sand field cricket) aggression in the morning (Nguyen and Stahlschmidt, 2019). These findings demonstrate that neurological pathways controlling behaviour can be under circadian control with both

the behaviour and control of such being exploitable by treatments to control pests such as *R. norvegicus*/*M. musculus*, crickets and *A. theophrasti*.

The reproductive behaviours of plants and animals show potentially exploitable circadian rhythms. Mammals' reproductive behaviour can rely on circadian clock genes with *Clk* and *Bmail1* mutant *M. musculus* being mostly infertile showing lack of responses to female pheromones, though the sensory organs responsible for such were functional (Kennaway, Voultsios and Boden, 2004, Mereness, 2016, Schoeller et al., 2016, Zhang et al., 2016, Wang et al., 2017, Pan et al., 2020 Sen and Hoffmann, 2020). Insects show clear time-dependent release and responses to sex-pheromones which are, similarly to mammals, lost in circadian clock mutants (Rosen, 2002 Rosen, Han and Lofstedt, 2003, Silvegren, Lofstedt and Rosen, 2005), with circadian rhythms in responses also shown, such as with *Ceratitis capitata* (Mediterranean fruit fly) (Sollai, Solari and Crnjar, 2018). Circadian rhythms interacting with sex pheromones provide an important opportunity for the timing of pheromone related treatments for the attraction and aversion of species. Also being useful for the disruption of mating, including with sterile insect technique (SIT) (Ikegawa et al., 2021, Bull and Gomulkiewicz, 2022). The disruption of circadian rhythms themselves can also directly produce infertility in species (Giebultowicz et al., 1989, Bebas, Cymborowski, Giebultowicz, 2001, Schoeller et al., 2016). Oviposition rhythms have also been shown, such as with *D. melanogaster, P. xylostella* and other lepidopteran pests, demonstrating times with significantly increased oviposition, which may be used to inform treatments most effective against the eggs of pests (Bell, 1981, Pivnick et al., 1990, T, Dass and Sharma, 2008). This may also be timed with emergence from egg or pupae which can show circadian rhythms (Tao et al., 2017, Mark et al., 2021) and may be when certain pest species are most vulnerable to treatments, similar to during ecdysis which may also have an exploitable rhythm (Lenaerts et al., 2017).

Plants use circadian rhythms to time the release of volatile signals also, as discussed previously with plant-pollinator networks (Fenske et al., 2015, Atamian et al., 2016, Bloch et al., 2017, Fenske et al., 2018), presenting opportunities for interacting treatments at certain times of day to be more effective. Seeds show circadian regulation and, similar to potential weakness during insect emergence, seedlings are potentially more vulnerable to treatments after end of dormancy and emergence from soil (Zhong et al., 1998, Casanova and Brock, 2000, Kim, Kim and Kim, 2013). Hatching, growth, timing of sexual maturation and behavioural development can all be significantly affected through circadian disruptions in animals (Minis and Pittendrigh, 1968, Reiter, 1980, Foster, Ebling and Claypool, 1988, Morse et al., 2003, Yadav, Thandapani and Sharma, 2014, Smarr et al., 2017). These factors may offer pathways for the disruption of pest species fitness, potentially through the timing of treatments to interact with or interrupt behaviours, reproductive activities and developmental processes, subsequently helping to control pest populations.

1.2.5 Limitations: Species-specific clock-independent rhythms and arrythmia

Though aspects of species physiology show both circadian rhythms and forms of circadian clock regulation, many studies have shown clock independent functions and or arrhythmia. Lack of maintenance of rhythms into constant conditions demonstrates that activity seen may be in direct response to zeitgebers rather than entrained by such through circadian clocks.

Certain species can lack rhythmic behaviour where there are few pressures that favour dark or light phase activity, shown in Lemuridae species which express cathemerality, being active across periods of both dark and light phase (Tattersall, 2008, LaFleur et al., 2014). As omnivores (Eppley, Ganzhorn and Donati, 2015), there are potentially more numerous food sources available to such species across both the day and night rather than selecting

for activity in either singularly. Research has shown arrhythmic behaviours may be widespread, with feeding behaviour shown in lepidopteran species such as *Danaus plexippus*, *Chloridea virescens* and *Manduca sexta* (Monarch butterfly, Tobacco budworm and Tobacco hornworm) being arrhythmic, which may be advantageous to species adapting to numerous zeitgebers simultaneously (Reynolds, Yeomans and Timmins, 1986, Niepoth et al., 2018). Certain mammalian species activity and organs also show no circadian regulation, such as *Lynx rufus* (Bobcat) and mammalian testes, with redundant pathways that can maintain function without circadian clock control. This can also be seen within insects, such as with certain fat body pathways (Kavanau, 1971, Morse et al., 2003, Boden and Kennaway, 2006, Erion et al., 2016). The lack of rhythmicity in certain physiological pathways present a barrier to the application of circadian based enhancement of treatments, however, interactions with circadian clocks are often found and the absence of rhythms in certain pathways is not consistent between species. Therefore, investigating species and pathway-specific activity rhythms may be required.

However, the potential need for highly specific analyses presents an additional barrier to the successful implementation of circadian rhythm informed management strategies. As discussed throughout this review, different species have differing activity rhythms and pathways, with varying interactions with circadian clocks. Therefore when circadian rhythms are found, it may be unlikely that findings can be applied to more than one species (Bloch et al., 2013, Muller, Korff and Davis, 2014). In addition, within closely related species there can be notable differences such as with *P. interpunctella* and *Ephestia kuehniella* (Mediterranean Flour Moth), both Pyralid moths, however showing opposite dawn and dusk calling rhythms with differing maintenance of rhythms into constant conditions (Zavodska et al., 2012). Also, within the mole rat superspecies *Spalax ehrenbergi*, genotypes were found relating to both circadian rhythmic and arrhythmic activity recordings from such (Ben-Schlomo, Ritte and Nevo, et al., 1995). Differences

between male and females of a species have also been found (Bertossa et al., 2013, Zavodska et al., 2012, Krizo and Mintz, 2015), moreover, such differences can be indiscriminate such as with *M. unguiculatus* where a mix of diurnal and nocturnal rhythms were found across sexes (Refinetti, 2006). Specific chronotypes have been found within species also, adding a further level of complexity (Refinetti et al., 2016). In spite of this, pest species-specific research is likely to increase, due to the increasing pressure to reduce pesticides while climate change worsens pest damage and crop yields (Cribb, 2010, Deutsh et al., 2018, Savary et al., 2019, Wagner et al., 2021), providing further opportunities to discover exploitable circadian rhythms.

In the natural environment numerous variable zeitgebers may be influencing the entrainment of circadian clocks and therefore interact with species-specific expression rhythms of pathways and behaviours (Refinetti, 2007, Pszczolkowski, 2008, Block et al., 2013). These cannot be precisely recreated in lab environments and though field/semi-field studies improve upon this, they may not account for the variation of certain pest species' ranges as many are highly invasive (Furlong, Wright and Dosdall, 2012, Panter et al., 2019, Shaw, Fountain and Wijnen, 2019). Therefore, recorded rhythms in treatment susceptibility and resistance may not be translatable to commercial implementation. The effect of microbiome and microbial communities established in both lab and natural settings may also affect the regulation of detoxification pathways (Liu et al., 1991, Claus, Guillou and Ellero-Simatos, 2016). Moreover, the addition of chemical treatment regimes themselves may entrain certain regulatory processes (Gillman et al., 2019), changing either endogenous or previously observed rhythms. However, in addition to the increase in pressure for the control of pest species and the rise of precision agricultural technologies (Strickland, Ess and Parsons, 1998, Natikar, Balikai and Anusha, 2016, Barnes et al., 2019), advancements in circadian rhythm analysis, such as through transcriptomics,

imaging and modelling (Currie et al., 2005, Dakhiya and Green, 2019, Roman et al., 2019, Tanigaki et al., 2019), will help allow for the exploitation of circadian rhythms for IPM.

1.2.6 Integration of circadian rhythms with integrated pest management

IPM aims to address the concern of pest population growth and spread of resistant strains via both environmentally and economically beneficial treatment pathways (Kogan, 1998, Ehler, 2006). The increasing severity of pests likely couples with the increasing spread of their pesticide resistances and, much like has been shown with antibiotic resistant bacterial spread, the more pesticides are used the more likely resistant strains of pests are to appear. The integration of multiple forms of treatment, a crucial part of IPM strategies, can also lower the chance of resistance gain as there isn't selection for a singular trait for pest survival (Llor and Bjerrum, 2014, Hawkins et al., 2019, Jorgensen et al., 2020). The harnessing of physiological pathways along with natural toxins and enemies is an important opportunity to disrupt target species without causing broad damage to environments through non-specialised toxic compounds, like those used in many pesticides (Nicolopoulou-Stamati et al., 2016, Hill, Macfadyn and Nash, 2017). This avenue of research in addition to typical pesticide use is where understanding species circadian rhythms and the precise timing of treatments could significantly increase efficacy, saving both economic costs and damage to the environment.

1.2.6.1 Biological toxins

Use of 'natural' toxins are a common alternative in place of the most frequently used pesticides and herbicides causing high environmental damage, such as glyphosates, neonicotinoids and firpronil, with the latter two making up 1/3 of the global insecticides used (Simon-Delso et al., 2014, Kanissery et al., 2019). *Bacillus thuringiensis* toxin (*Bt*T) has been used to compliment pest management and as a pesticide since 1938 and has been effective at increasing treatment efficiencies (Lambert and Peferoen, 1992, Betz,

Hammond and Fuchs, 2000, Deist et al., 2014). However, with increasing resistance levels to *Bt*T found across a number of species (Tabashnik et al., 1997, Peralta and Palma, 2017), the timed use when such will have the greatest effect on pest species outcomes may help maintain its effectiveness or reduce volumes needed, reducing economic costs and possible spreading of resistance. Similar can be said for other natural toxins including plant-derived treatments such as flavonoids and azadirachtin which have potentially more specific interaction pathways with pest species, disrupting feeding behaviour in *D. melanogaster* and other insect pests (Morimoto et al., 2006, Qiao et al., 2013). Classes of similar toxic and antifeedant chemicals are being isolated from natural plant defence pathways (Souto et al., 2021) and their use within IPM, and in respect to circadian rhythms of feeding and detoxification, has the potential to significantly increase treatment efficacies with more minimal impacts on the environment. The addition of further chemical toxins however must go hand-in-hand with supplementary treatment pathways to ensure crop protection and help prevent the rise of resistance (Ehler, 2006).

1.2.6.2 Genetic disruption

Though many species have developed resistances to certain treatments, genetic manipulation through siRNA (small interfering RNA), that by design can be highly specific and interrupt physiological pathways, can overcome such (Cagliari et al., 2019, Yan et al., 2020). A study showed that siRNA *si-Pxace1* (small interfering *Plutella xylostella* angiotensin converting enzyme I) treatments in *P. xylostella* not only reduced survival and growth of larvae but significantly increased their sensitivity to chlorpyrifos (He, Sun and Li, 2012). Understanding how the expression of the affected pathway is regulated over time and the feeding rhythms of *P. xylostella* and other insect pest species could aid in the timed targeting of this currently non-commercial form of treatment to increase efficacy, leading to profitability when used on infested crops. The direct genetic engineering of crop species to express siRNAs and chemicals such as *Bt*T has also been

pursued in addition to selective breeding (Romeis, Meissle and Bigler, 2006, Yan et al., 2020). The expression of such pathways, including natural plant defences, may be influenced to be expressed at certain times of day via circadian regulation (Pieterse et al., 2012, Burow and Halkier, 2017) when target pests are most active or least able to mediate the effects of such (Shipp and Otton et al., 1976, Akhtar et al., 2002, Oishi et al., 2003, Zhang et al., 2021). Doing so would decrease overall costs to plant fitness as well as reduce potential changes to crop plants form and nutrition by not simply increasing overall abundance of secondary metabolites in crop plants (Barton, 2015, Bell et al., 2018), potentially making such GM crops more acceptable for sale. Circadian clock modified GM crops are already being investigated for their potential benefits for crop fitness in varying environments (McClung, 2021).

1.2.6.3 Pheromones and traps

The use of chemicals and changes to abiotic conditions may cause non-specific effects, including the disruption of both pest and non-target species reproduction (Longcore and Rich, 2004, Sauer et al., 2018, Dominoni et al., 2020, Zheng et al., 2021). Species-specific pheromones, such as sex pheromones, however present a more specialised treatment for altering both species entrainment and disruption of reproduction. Pheromone signalling has been shown as an effective pathway to reduce pest species prevalence on crop fields. Scent marking or calls during peak reproductive activity can interfere with pest reproduction or deter pests from target crops (Aubin, 1990, Wang et al., 2012, Osada, Miyazono and Kashiwayanagi, 2015, Verrier, Baudry and Bessa-Gomes, 2021). Pheromone lures have also been effective as bait traps, both disrupting reproductive behaviours and directly trapping pest species (Mottus et al., 1997, Mitchell, 2002, Ahmad and Kamarudin, 2011, Rath et al., 2021). The use of pheromone sprays over crops works both by distracting pests from finding reproductive partners as well as desensitising pests to true signals given off by such (Miller et al., 2006). These methods have been successfully implemented on a

number of pests including *P. gossypiella*, *P. xylostella* and *Eucosma sonomana* (Western pineshoot borer) (McLaughlin, Mitchell and Kirsch, 1994, Lykouressis, 2005, Gillette et al., 2006, Chen, Fang and Zhang, 2007). Through continued research into gene editing, transgenic crops have been made to express sex pheromones themselves, removing the need for spraying (Mateos-Fernandez et al., 2021). Trap crops are also being harnessed that naturally inhibit pest population spread on crops via volatile signals that attract pests more than the target crops and can decrease the survival of the pest (Badenes-Perez et al., 2014a, Gyawali et al., 2021). These plant-based methods, including the previously mentioned pheromone signalling treatments, can benefit from understanding the timing of pest species circadian rhythms in reproductive behaviours, sensitivity to volatile signals and what is entraining such. By timing treatments with pest species rhythmic responses, as discussed previously, treatment efficacy may be increased. Therefore, helping to mitigate the increased costs of such specific treatments (Baker, 2008), by lowering quantities needed to be effective and therefore allow for wider implementation into IPM, reducing environmental damage.

1.2.6.4 Sterile insect technique and biocontrol agents

Another transgenic technology used for pest management is the use of SIT that harnesses tools such as CRISPR-Cas9, or classically with irradiation of males, to generate infertile or deleterious strains that are released into wild populations, inhibiting pest population expansion (Dunn and Follett, 2017, Aumann, Hacker and Schetelig, 2020, Ikegawa et al., 2021, Bull and Gomulkiewicz, 2022, Xu et al., 2022). The timing of initial release may be important for the effectiveness of reproductive inhibition due to dispersion and survival rates of transgenic strains (Bellini et al., 2010, Bolton et al., 2019, Duarte et al., 2020, Shelton et al., 2020). Therefore, the precise timing of release against pest species reproductive and activity rhythms may significantly increase the efficacy of this treatment pathway, reducing the time and cost of the mass rearing of transgenic pests to achieve

similar levels of pest population inhibition. The release of what are known as biological control agents may benefit from similar considerations. Biological control agents are species that have been found to target certain pests, with the aim that during introduction natural ecosystems will be minimally affected (Stiling and Cornelissen, 2005, Baker, Green and Loker, 2020). However, there are famous examples of how this may not be the case with there now being many potential restrictions to implementation and factors preventing permanent establishment of biological control agent populations (Simberloff and Stiling, 1996, Sarfraz, Keddie and Dosdall, 2007, Schulz, Lucardi and Marsico, 2019, Shine, Ward-Fear and Brown, 2020). However, previous research has also shown ways to increase the effectiveness of biological control agents that can be cleared for release, such as with the previously mentioned *Bt*T (Schuler et al., 2003, Navik et al., 2019). In addition, many biological control agents can come from different environments with varying photoperiods and zeitgebers to the introduced regions, it therefore may be important for maintaining efficacy to match biological control agents with the circadian rhythms of pests to increase exposure and lead to further pest population reduction.

1.2.6.5 Entrainment

Further to the aforementioned IPM strategies, influencing entrainment pathways may be an avenue to increase the efficacy of treatments. As previously discussed, the mismatching of circadian rhythms alone can have significant effects on the outcome of species health and interactions with abiotic and biotic factors (Silvegren, Lofstedt and Rosen, 2005, Smarr et al., 2017, Fenske et al., 2018, Pickel and Sung, 2020). With both the genes and signals defining circadian rhythms becoming known, the timed application of chemical treatments or zeitgebers may be able to cause these deleterious effects, either directly inhibiting pest populations or complimenting the effectiveness of other treatments. Numerous environmental factors have been shown to affect the regulation of circadian rhythms and specific pathways, some of which are correlated with human waste such as progestins and

glucocorticoids (Abraham et al., 2010, Dunlap and Loros, 2017, Sauer et al., 2018, Zheng et al., 2021), therefore such factors are likely already commercially available. The maintenance of specific light and temperature regimes can also be harnessed, with temperature helping to entraining oviposition rhythms in *D. melanogaster* (Kannan et al., 2012) and light pulses potentially able to synchronize the timing of egg hatching in *P. gossypiella* (Minis and Pittendrigh, 1968), both informing times when pests may be most vulnerable to IPM treatments. Maintenance of light treatments on fields alone may be enough to prevent pest populations growing on crops, causing disruptions to activity (Shimoda and Honda, 2013) and fertility (Giebultowicz et al., 1989), in addition to potentially increasing efficacy of other IPM treatments via disruption of circadian clocks.

1.3 Origin and impact of the Diamondback Moth (*Plutella xylostella*) as an agricultural pest

1.3.1 Introduction to the Diamondback Moth

1.3.1.1 Lepidopteran pests

Lepidoptera as an order of insects includes all moths and butterflies (Scoble, 1992), many species of which are classified as pests, causing economic losses such as wood pests like *Zeuzera coffeae* (Red coffee borer) and *Endoxyla cinereus* (Giant wood moth) (Ahmad, 2017, Thurman, 2022), cloth pests such as *Tineola bisselliella* (Common clothes moth) (Plarre and Kruger-Carstensen, 2011) and most predominantly agricultural pests. Lepidopteran agricultural pests cause significant losses to farmers globally with climate change facilitating the range expansions of such pests (Bradshaw et al., 2016, Lehmann et al., 2020, Skendzic et al., 2021). The Diamondback Moth (*P. xylostella*) is one such pest and is perhaps the world's most economically impactful lepidopteran, costing the agricultural industry ~$5 billion annually (Zalucki et al., 2012, Li et al., 2016).

1.3.1.2 Identifying *P. xylostella*

P. xylostella is most identifiable by the diamond-shaped patterning that occurs down adults folded wings, most prominently seen in males where there is a strong contrast between light brownish 'diamonds' and dark brown outlines and edges of the wings, giving the common name of the Diamondback Moth. Females also maintain a similar pattern but with reduced contrast between shades of wing colour (Harcourt, 1956). Males will also have a relatively long orange slit at the end of the abdomen which opens to reveal the phallus for reproduction, while females have notably thicker abdomens lacking such a pronounced genital opening (Moriuti, 1986, Landry and Hebert, 2013). There is little difference between male and female length however, with both male and female wingspans equal to ~12mm (Landry and Hebert, 2013) and a body length of ~6mm (Philips et al., 2014). The presence of *P. xylostella* larvae can be identified by visibly distinct foliage damage where windowing occurs as newly hatched *P. xylostella* larvae act as leaf miners, consuming the inside of leaves leaving the waxy cuticle behind, afterwards skeletonising foliage as the larvae mature. The larvae once visible outside leaves are small and pale, >2mm in length, however, can quickly grow up to a recorded length of ~11mm, generally becoming greener over time (Harcourt, 1957, Philips et al., 2014). The larvae also demonstrate a distinctive avoidance behaviour when threatened, wriggling violently, often throwing themselves from leaf surfaces to avoid threats, potentially using silk threads to hang and move between areas of foliage (Sarfraz, Dosdall and Keddie, 2009).

1.3.1.3 *P. xylostella* life cycle

Once mated, female *P. xylostella* may be able to lay up to ~400 eggs, with various factors influencing fecundity (Sarfraz, Dosdall and Keddie, 2007, Sarfraz, Dosdall and Keddie, 2009, Sarfraz et al., 2011, Jaleel et al., 2017), laying mostly along ridges and the adaxial surface of leaves, though this may also depend on age and position of the leaves (Harcourt, 1956, Gupta and Thorsteinson, 1960, Talekar et al., 1994). The development of *P.*

xylostella eggs, like many *P. xylostella* life history traits, is significantly affected by temperature, with multiple studies showing temperature dependent egg hatching. At temperatures between 14-18°C one paper showed 100% egg survival with significant drop offs in such above 32°C and below 8 °C (Liu, Chen and Zalucki, 2002), while studies on development speed showing ~32.5°C reared eggs hatched fastest (Golizadeh et al., 2007, Ngowi et al., 2017). Hatched 1st instar larvae enter leaf tissues, burrowing inside and consuming the nutritious mesophyll layer of leaves, leaving the waxy cuticle behind which creates the distinctive windowing effects. Once ready, the larvae emerge as 2nd instar larvae and begin to skeletonise foliage reaching a 4th instar before pupating. The length of the larval life stage is highly dependent on temperature as well as the provision of food. As the temperature drops *P. xylostella* development rate drops with it, with studies showing increased time to pupation at lower temperatures, taking between ~5 days at ~32°C (11 days total to reach adulthood) and ~55 days at ~8°C, 11x longer (119 days total to reach adulthood) (Harcourt, 1957, Liu, Chen and Zalucki, 2002, Garrad, Booth and Furlong, 2015). The type of food provided can also affect length of time in larval stage along with final *P. xylostella* mass (Golizadeh et al., 2009, Sarfraz et al., 2011). During pupation, a pre-pupation stage occurs where *P. xylostella* larvae weave open lattice silk cocoons. The speed at which larvae form into pupae inside the cocoon is relatively short, taking less than a day up to 2, again dependent on temperature (Talekar and Shelton, 1993, Liu et al., 2002, Golizadeh et al., 2009). Time spent as a pupae and pupal mass can also vary significantly depending on temperature and food type (Liu et al., 2002, Sarfraz et al., 2011). Emerging adults feed on dew and possible sugar sources (Talekar and Shelton, 1993), being able to reproduce from day of emergence, with reproductive activity being concentrated at dusk and in response to host plant volatiles (Hillyer and Thorsteinson, 1969, Pivnick et al., 1990, Campos, Schoereder and Sperber, 2004). Oviposition peaks shortly after dusk (Pivnick et al., 1990) starting the cycle over again. Adult *P. xylostella* longevity can also

be affected by temperature, increasing as the temperature decreases, living up to ~8 weeks, with life stage specific temperature tolerances shown (Harcourt, 1957, Sivapragasam and Heong, 1984, Liu et al., 2002). This developmental dependency on temperature can be related to *P. xylostella's* inability to diapause. Some insect species react to changing environmental conditions by entering diapause where metabolism and development is suspended until conditions change (Denlinger, 2002, Anduaga et al., 2018). However, *P. xylostella* lacks this ability, unable to survive high latitude winters with clear distinctions being found between regions where *P. xylostella* can over-winter and ones that are invaded through seasonal migration (Ma, Ma and Yang, 2010, Zalucki and Furlong, 2011).

1.3.2 Migration and origin of *P. xylostella*

1.3.2.1 Distribution and migration of *P. xylostella*

Despite lacking the ability to diapause, *P. xylostella* has perhaps the largest range out of any lepidopteran, covering the planet in a cosmopolitan distribution, meaning they inhabit all continents bar Antarctica (Talekar and Shelton, 1993, Zalucki and Furlong, 2011). This range is split between a year-round population and a seasonally expanding range that expands to higher latitudes as summers allow *P. xylostella* development and the growing of crops *P. xylostella* is attracted to (Talekar and Shelton, 1993, Justus and Mitchell, 1996, Zalucki and Furlong, 2011, Chapman et al., 2002). *P. xylostella* is highly proficient at migrating to seasonal regions, harnessing air currents to travel up to ~500km/night sometimes forming migratory swarms, invading regions quickly and heavily affecting farmers (Harcourt, 1957, Chapman et al., 2002, Reynolds et al., 2010, Wainwright et al., 2020). Therefore, attempts to restrict human aided *P. xylostella* transportation would likely be insufficient to stop population spread. Migratory phenotypes of *P. xylostella* may arise due to signals from host plants, such as volatiles indicating low nutrition or plant health in combination with other host plant signals acting as attractants (Reddy and Guerrero, 2001,

Campos, Schoereder and Sperber, 2004). These phenotypes have desirable traits for migratory journeys such as smaller bodies and delayed reproductive maturity, though it remains unclear how they survive cold temperatures in certain air currents (Talekar and Shelton, 1993, Campos, Schoereder and Sperber, 2004). There is little evidence for return migrations as winter months begin, demonstrating that in most circumstances winters cause expanded populations to die off, however, some research has shown limited migration towards lower latitudes (Wei et al., 2013).

1.3.2.2 Attraction of *P. xylostella*

Attraction to target host crops plays an important role once *P. xylostella* invades new regions, where it can infest up to 92% of brassica farmer's fields (Uthamasamy et al., 2011). *P. xylostella* is attracted to brassica crops through the sensing of a unique secondary metabolite to Brassicaceae called glucosinolate (Feeny, 1977). Glucosinolates acting as a strong attractant for *P. xylostella* is likely an adaption to the competitive advantage *P. xylostella* has on plants that produce glucosinolate compared to other insect herbivores. This advantage comes from a unique specialised protein *P. xylostella* possesses called glucosinolate sulfatase. This inhibits a key brassica defence mechanism known as the MOB, making *P. xylostella* a brassica feeding specialist (Ratzka et al., 2002, Kumar, 2017). *P. xylostella* senses glucosinolates via olfactory receptors in the antennae, potentially relying on numerous sensillum, including trichoid sensilla, *P. xylostella* possess to detect sex pheromones and other plant volatiles. Similar structures are found on larval *P. xylostella* mouth parts, demonstrating a pathway for larval attraction responses (Yan et al., 2014, Li et al., 2018). A chemosensory protein, *Pxyl*CSP11 (*Plutella xylostella* Chemosensory protein 11), may mediate these responses to volatiles and pheromones, also being significantly more abundant in males that may use such to help track female sex pheromones (Fu et al., 2020). Other key sensory molecules have also been found in *P. xylostella* mouth parts (*Pxyl*Gr34 (*Plutella xylostella* gustatory receptor 34)) that help

mediate responses to plant host defence volatiles, with brassinolide hormones being found to inhibit feeding through these pathways (Yang et al., 2020). However, glucosinolate has been shown to be the key attractant and stimulant for oviposition and feeding behaviour and is likely the predominant molecule used for target host recognition by *P. xylostella* (Gupta and Thorsteinson, 1960, Justus and Mitchell, 1996, Sun et al., 2009, Badenes-Perez et al., 2014b). *P. xylostella* is not attracted to one specific form of glucosinolate but responds to the overall composition of such and quantity of other secondary metabolites (Li et al., 2000, Robin, 2017). The presence of non-Brassicaceae plant volatiles during development can also 'train' *P. xylostella* females to trigger oviposition responses to such (Zhang et al., 2007). This may also be important as adult *P. xylostella* can leave pheromones behind on plants that can act as an attractant to food sources for larvae (Zhu et al., 2016), therefore, such responses may lead to host range expansions in the lab and natural environments.

1.3.2.3 Phylogeny of *P. xylostella*

P. xylostella is the only global pest in its genus with other *Plutella* species being found in more restricted overlapping ranges such as in the Americas, Europe and Australia. These species also feed on Brassicaceae species, however not at the same scale of *P. xylostella* (Hodges, 1983, Heppner, 1984, Perry et al., 2018, You et al., 2020). The *Plutella* genus belongs to the Plutellidae family and diverged from the *Eidophasia* genus ~24.5 million years ago and is in the superfamily Yponomeutoidea (~Ermine moths), which arose ~117 million years ago (Wahlberg, Wheat and Peria, 2013, Edde, 2022). *P. xylostella* has evolved as a specialist brassica feeder through the adaption of specialised glucosinolate sulfatase genes that disarms the MOB unique to Brassicaceae (Ratzka et al., 2002, Kumar, 2017). It has been shown that *P. xylostella* is most successful feeding on host plants within the Brassicaceae order, though may be able to feed successfully on non-host plants (Lohr and Rossbach, 2001, Rossbach, Lohr and Vidal, 2007, Sarfraz, Dosdall and Keddie, 2012).

1.3.2.4 Origin of *P. xylostella*

There may be problems controlling *P. xylostella* pest populations due to its unclear origin. It is important to understand where a pest species originates as it may be key to uncovering natural systems that have developed to control pest species populations and keep such in balance within an ecosystem (Wohlgemuth, Solan and Godbold, 2017). It has however proved difficult to identify the exact origin region for *P. xylostella*, due in part to its global distribution and ability to follow wherever brassicas are grown (Wei et al. 2013). This confusion on point of origin has potentially limited the effectiveness of harnessing natural enemies (Biological control agents) in pest management (Azidah, Fitton and Quicke, 2000). Initial research hypothesised a European origin, likely due to historic brassica agriculture in the region (Hardy, 1938, Harcourt, 1956). This led to Europe being the main region of investigation for *P. xylostella* natural enemies, however this proved relatively unsuccessful. This caused questioning of the theory of *P. xylostella* spread via Dutch colonizers introducing cultivated brassicas to South Africa as food near the end of the 17th century, but also as a cash crop to sell onto Asia (Kfir, R. 1998). This had demonstrated a vector of human aided *P. xylostella* range expansion through the movement of infested crops, however, these two regions were later conversely identified as likely regions of *P. xylostella* origin.

South Africa was searched for natural enemies of *P. xylostella* multiple times (Ullyett, 1947, Kfir, 1997) and has provided a selection of at least 22 parasitoids, this number including at least one parasitoid for each stage of *P. xylostella*'s life cycle (Kfir, 1998). In addition, the south African region has the highest diversity of wild brassica species anywhere in the world (Jordaan, 1993, Kfir, 1997, 1998). Another factor to consider is one of the few currently used biological control agents against *P. xylostella*, *Diadromus collaris* (an Ichneumonid wasp), presents as asexual everywhere except South Africa (Kfir, 1998), this points towards a South African origin due to asexual *D. collaris* forms arising

from sexual forms (Dougherty, 1955, Kfir, 1998). Due to the number of natural enemies, damage caused by *P. xylostella* in this region is lower than in other similar climates (Kfir 1998). These factors demonstrate South Africa as a likely origin site for *P. xylostella*, in part due to the large diversity of natural enemies being highly unlikely to have arisen within the space of 300 years with the arrival of Dutch colonists if the European origin theory is accepted (Whitfield, 1998). Modern genetic analysis has also pointed to the south African region as the most likely site of origin (Juric, Salzburger and Balmer, 2016). However, though this region hosts the largest diversity of brassica species, all species within the *Plutella* genus are brassica feeding specialists, found all over the world, most commonly in the Americas (Hodges, 1983, Heppner, 1984), therefore, diversity of brassicas may not be strong evidence for the *P. xylostella* origin region. Moreover, assuming 'diversity of natural enemies equates to origin', also places south China as a possible origin site of *P. xylostella* as it was found to have 8 parasitoids and 7 hyperparasitoids endemic to the region, again, likely too many to occur since human aided range expansion (Liu *et al*. 2000). Further genetic analysis however shows that certain parasitoids in this region, such as *Cotesia vestalis* (a parasitoid wasp), rapidly adapted to historic *P. xylostella* expansion (Ke et al., 2019).

The localisation to one specific origin region for *P. xylostella*, particularly before supposed human aided range expansion, may be ineffective due to the abilities *P. xylostella* possess for long range seasonal migrations, as previously discussed (in 1.3.2.1), masking such. In specific regions with seasonal migrations such as in China, *P. xylostella* populations did not show distance correlated genetic differences, however core year-round populations may tend to be more diverse (Wei et al., 2013). Even when host plants were maintained year-round, *P. xylostella* populations still oscillated seasonally (Campos, Schoereder and DeSouza, 2006). These findings may be applied to multiple regional populations of *P. xylostella* around the world where swarms can undergo rapid and distant dispersals without

the need for human transportation, including invasions of high artic islands, with similar likely to become more common as the climate changes (Chapman et al., 2002, Coulson et al., 2002). Smaller scale population changes are therefore potentially part of large-scale regional movements/events in *P. xylostella* migration. Causal processes of *P. xylostella* migration may be on a global scale with regional invasions being parts of global *P. xylostella* population movement ecology (Campos, Schoereder and DeSouza, 2006). These factors likely feed into the difficulty of pinpointing a singular site of origin for *P. xylostella*, with recent genome analysis now pointing to a South American origin (You et al., 2020), with previous studies showing similar parasitoid diversity to the other candidate sites of origin, with such again being unlikely to have developed recently (Guilloux et al., 2003). Nevertheless, *P. xylostella* is now a global pest and is affecting brassicas both wild and farmed worldwide.

1.3.3 *P. xylostella* feeding, resistance and regulation

1.3.3.1 *P. xylostella* brassica feeding

P. xylostella is a brassica feeding specialist and through both this and a number of advantageous traits has become perhaps the most damaging lepidopteran pest, potentially causing more than $5 billion of damage annually as infestations worsen over time (Zalucki et al., 2012, Li et al., 2016). Brassica (cruciferous) crops include numerous food crops such as cabbage, turnip, broccoli and kale, but also includes seed oils from rapeseed which is an increasingly important cash crop with ~25.7 million tons produced from 2011-2015 compared to ~11.8 million tons from 1996-2000 (OECD, 2016). The losses of these crops heavily affect developing regions which rely on cruciferous plants as an important source of vitamins and minerals in many cultural diets (Shelton, 2004, FAO, 2013, Muimba-Kankolongo, 2018). Additionally, large scale agriculture can be heavily affected, where in China over 2 million ha of brassicas are lost annually with a 61% increase in losses per ha

since the 1990s (Li et al., 2016), with some regions in Asia suffering significant losses due to *P. xylostella* to up to 92% of brassica farmers (Uthamasamy et al. 2011).

1.3.3.1.1 Outcompeting the MOB

Perhaps the foremost reason for *P. xylostella*'s level of damage to brassicas is their ability to disarm the Brassicaceae unique defence mechanism, the MOB. The MOB occurs via the secondary metabolite glucosinolate interacting with plant myrosinase (Fenwick and Heaney 1983, Singh, 2017, Wang et al., 2019). Increased levels of glucosinolate and subsequent MOB reactions would typically limit the amount of feeding herbivores can carry out due to the build-up of herbivory deterrents (Feeny, 1977), however, *P. xylostella* can feed unrestrained by such toxic product build up. *P. xylostella* harnesses glucosinolate sulfatases (GSSs) that intercept glucosinolate before it forms a glucosinolate-myrosinase complex. GSS removes a sulphate group from glucosinolate, forming desulpho-glucosinolate, which can be safely excreted without binding to released myrosinase shown in Fig. 1.5 (Ratzka et al., 2002).

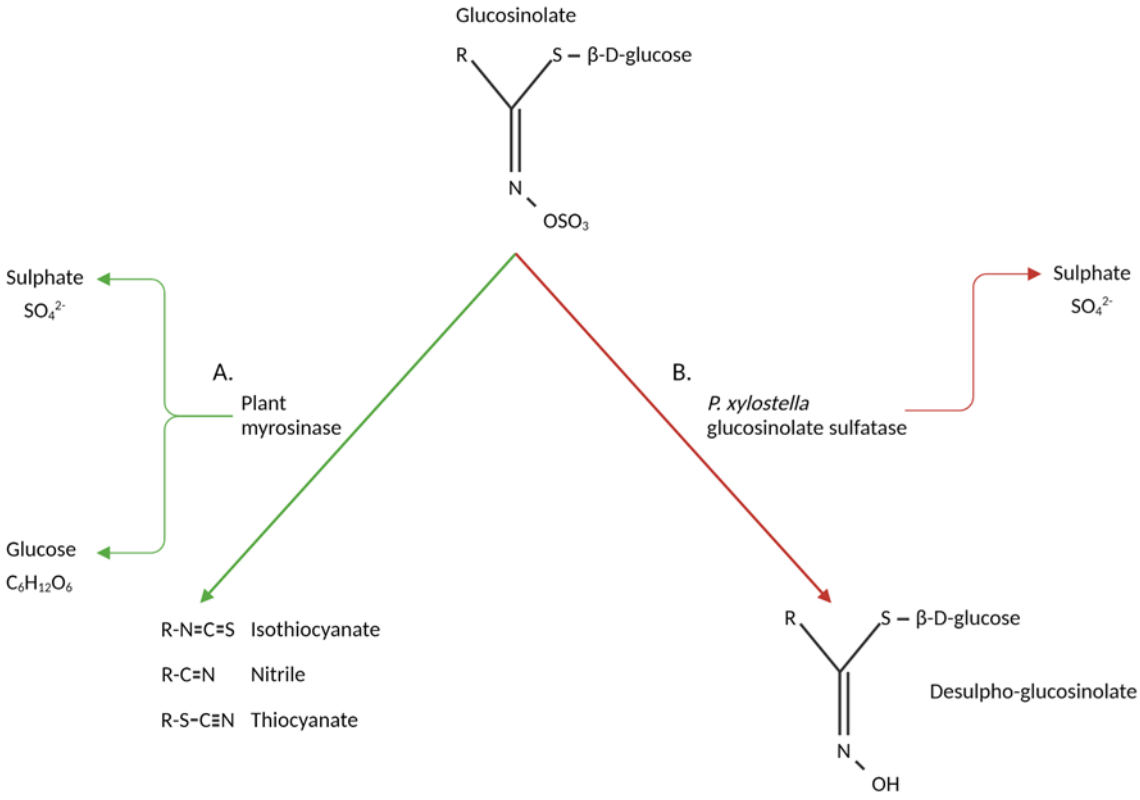


Figure 1.5 MOB and glucosinolate sulfatase reaction.

A. shows MOB pathway where plant myrosinase binds to glucosinolate removing a sulphate and glucose group creating an unstable intermediary product that breaks down into toxic hydrolysis products isothiocyanates, nitrile and thiocyanate. B. shows how *P. xylostella* glucosinolate sulfatase intercepts glucosinolate before it binds with plant myrosinase, removing a sulphate group generating a stable product, desulpho-glucosinolate, that can be safely excreted. Adapted from Ratzka et al., 2002.

The activity of these GSSs is not completely unique to *P. xylostella*, yet still provide a selective advantage over other insect herbivores (Robin et al., 2017). Specifically, it was found that intact indolic glucosinolate and products produced from aliphatic glucosinolates acted as key attractants (Sun et al., 2009), showing these as potential sources of competitive advantage. In contrast, recent studies have shown that no specific group of glucosinolate is preferred during *P. xylostella* feeding and instead the overall profile of glucosinolates produced in plants affects feeding choice. In addition, the total amount of glucosinolates do not show effects on preferences (Robin et al., 2017). Other research has

also shown the quantity of glucosinolates from either categories of such do not affect feeding preferences (Arany et al., 2008) and that it is the quantity of myrosinase that has the highest effect on *P. xylostella* feeding (Li et al., 2000). Recent research has also shown that higher levels of glucosinolate increase attraction and may increase host plant suitability (Badenes-Perez et al., 2014a, Badenes-Perez, Gershenzon and Heckel, 2020).

1.3.3.2 Glucosinolate sulfatase genes

P. xylostella is susceptible to glucosinolate hydrolysis products, so therefore must rely on GSS for disarmament of the MOB for unrestrained feeding on brassicas, likely leading to the lower preference for plants rich in myrosinase as this may compete with GSSs (Li et al., 2000). The gene family responsible for MOB disarmament has been investigated, with a full genome sequence also showing *P. xylostella* has significant expansions to gene families involved in both the perception and detoxification of plant secondary metabolites (You et al., 2013). This likely occurring in conjunction with each other as host shifts occurred in *P. xylostella's* phylogenetic history as brassica feeding specialisms arose. Expression levels of likely GSS genes and regulators was high in late-stage larvae, involving SUMF1 (Sulfatase modifying factor 1) which is required for sulfatase protein activity (Buono and Cosma, 2010, You et al., 2013). A paper by Ma et al. (2018) localised the expression of these highly expressed sulfatase genes both temporally and spatially in *P. xylostella*, showing clear identification of 2 GSSs, out of 12, that were predominantly expressed in the midgut of 3rd and 4th instar *P. xylostella* larvae. *PxylSumf1a* (*Plutella xylostella sulfatase modifying factor 1 alpha*), a copy of the SUMF1 coding gene, was highly correlated with the expression of these two genes, named in Ma et al., 2018 as *PxylSulf2 (Plutella xylostella sulfatase 2)* (*Sulf2)* and *PxylSulf3* (*Sulf3*) (Ma et al., 2018). A more recent paper detailed the arylsulfatase gene family GSSs are located in, identifying multiple duplication events including an inversion. Located within the *Sulf-C* (*Sulfatase C*) gene cluster, 3 inverted GSS genes were identified, *PxGSS1*(*Plutella xylostella*

glucosinolate sulfatase 1) and *PxGSS2* arising from a more recent duplication event and *PxGSS3* from an older event. *B. mori*, another widely researched moth, has a *Sulf-C* duplication event but without gain of glucosinolate interactions. *PxGSS1* appears to have gained universal glucosinolate interactions while *PxGSS2* is more restricted to long chain Met originating glucosinolates and *PxGSS3* for Phe and Trp glucosinolate (Heidel-Fischer et al., 2019). This mix of glucosinolate specificities correlates with the previous findings that *P. xylostella* MOB disarmament, and subsequently attraction and feeding preference, is not adherent to any specific group of glucosinolates but responds to the overall profile of glucosinolate content within host plants (Robin et al., 2017). These papers were also compared to reconcile differences in GSS naming schemes. The findings using gene sequences and alignment from both papers to construct a phylogenetic tree using PRESTO (Shank, Weaver and Pond, 2018) demonstrated that Heidel-Fischer et al.'s *PxGSS1*, *PxGSS2*, *PxSulfB* and *PxSulfD* correlate to Ma et al.'s *PxylSulf2*, *PxylSulf3*, *PxylSulf4* and *PxylSulf1* respectively, shown in Table 1.1.

Table 1.1 *P. xylostella* glucosinolate sulfatase genes naming schemes

Names assigned to GSS genes from both Ma et al. and Heidel-Fischer et al. listed against each other, showing equivalent gene name in each row between the two papers as shown by sequence alignment.

Ma et al., 2018	Heidel-Fischer et al., 2019
PxylSulf2	*PxGSS1*
PxylSulf3	*PxGSS2*
PxylSulf4	*PxSulfB*
PxylSulf1	*PxSulfD*

1.3.3.3 Pesticide resistance

These findings demonstrate *P. xylostella* genetics have likely undergone strong positive selection for the detoxification of brassica secondary metabolites, in addition, *P. xylostella* has been shown to have a diverse genome that may rapidly adapt to environmental changes (You et al., 2013). Heidel-Fischer et al. calls these rapid genetic adaptations "concerted neofunctionalization", originating from the findings that a high frequency of duplicated genes are positively selected for new roles within *P. xylostella*, while previous functions are lost or maintained elsewhere in the genome (Heidel-Fischer et al., 2019). Concerted neofunctionalization may be found to be evident within *P. xylostella's* other detoxification pathways which can be co-opted to resist the effects of pesticides (Tao et al., 2012), as *P. xylostella* rapidly gains resistance against chemical treatments. This includes being known as the first pest to become resistance to the pesticide responsible for the silent spring movement DDT (Ankersmit, 1953), and more recently *Bt*T (Tabashnik et al., 1990, Tabashnik et al., 1997). The collection of pesticide resistance genes *P. xylostella* possess in addition to the co-option of secondary metabolite detoxifying genes are likely key factors pesticides have so far proven ineffective at sustainably controlling *P. xylostella* pest populations, with resistance found to all currently used artificial chemical pesticide families (Heckel DG, 1999, You et al., 2013, Jaleel et al., 2020). An example of rapid resistance gain was shown with a *P. xylostella* strain resistant to the pesticide chlorantraniliprole collected from the wild and reared in the lab under exposure to such for 1 year, gaining 2157-fold resistance to chlorantraniliprole compared to naïve susceptible strains. A point mutation was highlighted in a ryanodine receptor gene along with general increases in detoxification pathway activity in this selected for strain, therefore selection for chlorantraniliprole resistance may lead to cross-resistances (Kang et al., 2017). Other gene products important in chlorantraniliprole resistance have been found affecting GST detoxification pathways, such as GSTu1 (Glutathione S-transferase u1) (Zhu et al., 2021).

Abamectin resistance have also been researched, showing that multiple genes may be involved in abamectin specific pathways rather than upregulating general detoxification pathways. Compared to the more recessive resistance genes for abamectin (Liang, Gao and Zheng, 2003), deltamethrin resistance genes are more dominant, though also lack cross-resistance (Balasubramani, Sayyed and Crickmore, 2008). Gene products responsible for resistance to *Bt*T found in wild populations, *Px*ABCC2 (*Plutella xylostella* ATP-binding cassette transporters C2) and *Px*ABCC3, have also been identified. Additionally, it is mutations in both genes combined that provides significant increase in resistance to *Bt*T (8000-fold in both compared to 4-fold individually) (Liu et al., 2020). The provision of pesticide resistance may also occur through *P. xylostella* gut microbiota, with *Enterococcus* species presence in the gut enhancing resistance to commonly used pesticides, however other species in the gut such as *Serratia* species decreased resistance (Xia et al., 2018).

Life history traits may compound these issues. Alongside rapid seasonal migration and field invasions, *P. xylostella* can cycle through multiple generations in a single season, allowing for multiple amplifications of population once food sources are found. Due to this, multiple life stages can occur within a single crop field at once, potentially forcing the use of broad-spectrum treatments, as if one part of the life cycle is unaffected, the crop can be re-infected (Crafford and Chown, 1990, Talekar and Shelton, 1993). An example of these issues is with 1st instar larvae being offered higher protection from pesticides and non-specialised parasitoids and predators while inside plant leaves (Talekar and Shelton, 1993, Hermansson, 2016). Behavioural changes may also aid in the avoidance of pesticides where wild *P. xylostella* populations have been shown to lay eggs near the base of crop plants, potentially reducing the exposure to pesticide sprays (Sarfraz, Dosdall and Keddie, 2005)

1.3.3.4 Host range expansions

Crop rotation in combination with the drying of land and removal of crop residues has been shown to prevent *P. xylostella* reservoir populations from forming, due to their usually narrow host range (Sayyed, Rizvi and Alvi, 2002), however, there is evidence of *P. xylostella* having an incomplete evolutionary host range expansion, shifting its host range to include sugar snap peas (*Pisum sativum*) in Kenya (Knolhoff and Heckel, 2011, Henniges-Janssen et al., 2011a,b). This comprises a host shift to a different plant order (Fabales) from brassicas (Brassicales). This level of host change requires the adaptation of numerous mechanisms, however pea strain *P. xylostella* do not have significantly reduced larval weight or survivorship on brassicas while able to survive on the novel host, with non-adapted *P. xylostella* strains having very low survivorship on pea plants (Lohr and Rossbach, 2001). Though the ability to survive on pea hosts is genetic, preference for either brassica or pea is not fixed, with pea strain *P. xylostella* primed on cabbage choosing such over pea substitutes in choice experiments (Henniges-Janssen, Heckel and Groot, 2014). This kind of priming has been used to select for pea adapted strains in labs, showing significantly increased survivorship after 4 generations of pea plant exposure, demonstrating *P. xylostella's* adaptability (Lohr and Kahuthia-Gathu, 2002). Similar has been shown to occur through plant volatile exposure priming *P. xylostella* over its development to shift plant hosts (Zhang et al., 2007, Zhu et al., 2016). However, this adaptation came at a cost of smaller larvae with longer development times and increased susceptibility to *Diadegma* wasp parasitoids on pea plants (Lohr and Rossbach, 2001). This host range expansion still sets a concerning precedent, given *P. xylostella*'s level of resistance to chemical control measures that otherwise suppress its competitors and natural enemies on crops. As *P. xylostella* can shift in host plant clades, there is the potential to infect other crop families which have been freed from competitors by chemical treatments *P. xylostella* are resistant too. Therefore, *P. xylostella*'s ability to outcompete other insect

herbivores on brassicas will be less a reason for its success in modern agriculture than its resistance to chemical treatments on crops already cleared of competition. This may be demonstrated by comparing with *P. xylostella*'s closest relative, *Plutella australiana,* which is also a specialist brassica feeder but up to ~300x more susceptible to certain pesticides and so only accounts for 10% of *Plutella* populations on crops local to *P. australiana* ranges (Perry et al., 2018).

1.3.3.5 Lepidopteran circadian clock

Circadian rhythms and the manipulation of the circadian clock can influence behaviour of pest species, including susceptibility to possible control measures (Meireles-Filho and Kyriacou, 2013, Suszczynska et al. 2017). Circadian clocks can have wide-reaching effects on organism's physiology and are present in most life, including pest species as previously discussed (in 1.2), enabling species to take advantage of the ~24h rhythms in both abiotic and subsequent biotic factors (Pittendrigh, 1993). The molecular function of circadian clocks has been demonstrated using several model organisms with a significant volume of research on *M. musculus* and *D. melanogaster* circadian clocks as mammal and arthropod models respectively, shown in Fig. 1.2. Though certain genetic components and interactions differ between these two models, many are highly conserved with the model of function through TTFL consistent between both (Chang and Reppert, 2001, Rosato, Tauber and Kyriacou, 2006). *D. plexippus* has been researched extensively and has become a model for lepidopteran species circadian clocks shown in Fig. 1.6, however much is still inferred from the *D. melanogaster* model (Kyriacou, 2009).

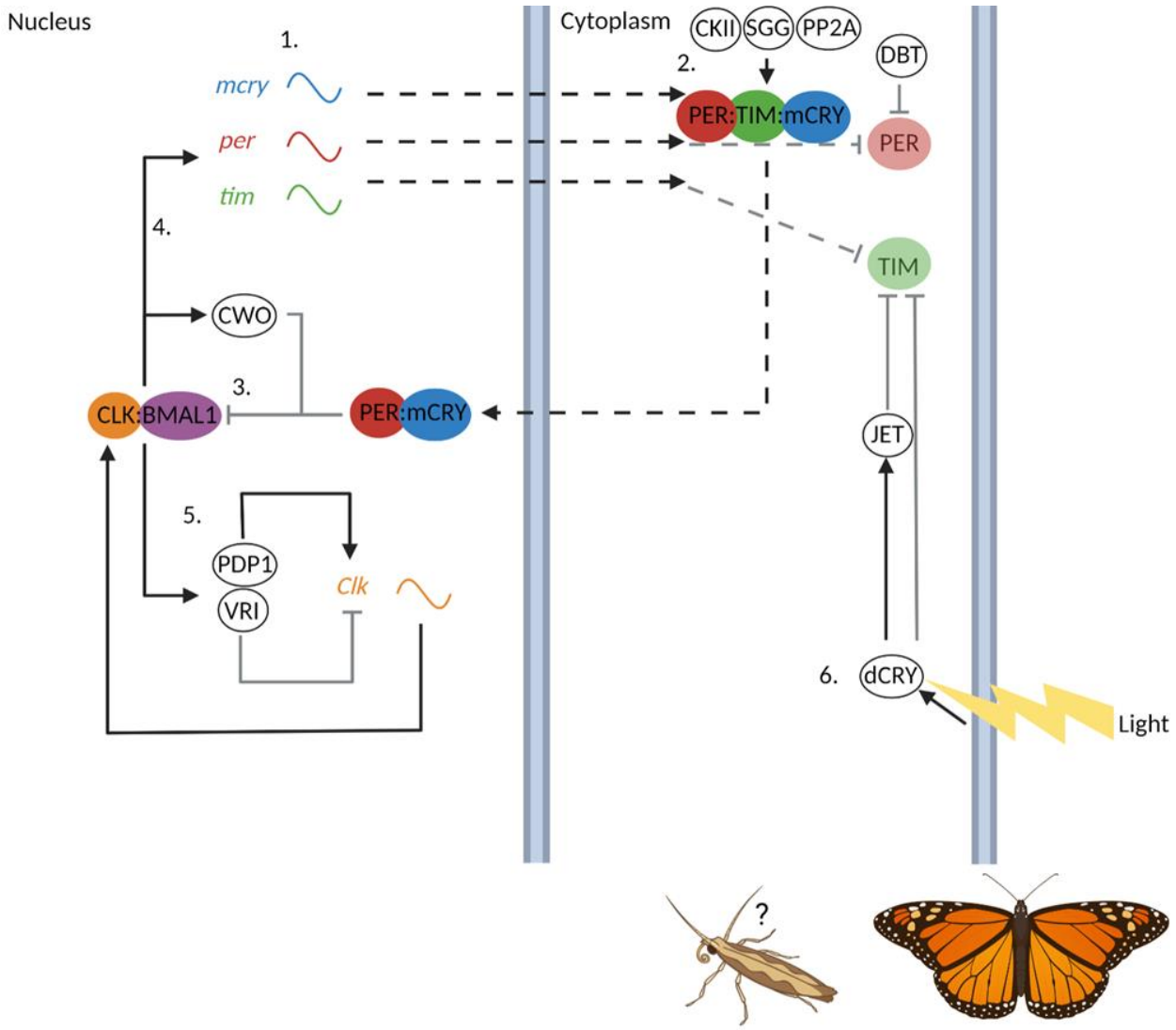


Figure 1.6 The lepidopteran molecular circadian clock.

Solid black arrows equate to promotion, flat head grey arrows indicate inhibition and hashed arrows equate to molecular movement specifically. Lower case italics indicate genes with lines indicating transcripts next to such, upper case in circles indicates proteins with joined proteins indicating complexes. Colour coding for genes and circles for proteins are also used to highlight key rhythmic circadian clock genes. Faded boxes also show degradation of proteins.

The lepidopteran molecular circadian clock is predominantly modelled using *D. plexippus* studies with *D. melanogaster* circadian clock genes found in lepidopterans inferred in the model as they have been shown to function in *D. melanogaster* previously. 1. PER, TIM and mCRY is produced and accumulates in the cytoplasm as part of the core circadian molecular oscillator. 2. A PER:TIM:mCRY complex is formed, stabilised by CKII, SGG and PP2A to allow for nucleus entry (Martinek et al.,

2001, Akten et al., 2003, Sathyanarayanan et al., 2004, Zhu et al., 2006). However DBT promotes the degradation of PER (Price et al., 1998) and JET for TIM (Koh, Zheng and Sehgal, 2005) and it is currently unclear how mCRY is broken down. 3. PER stabilizes mCRY for nucleus entry leaving TIM behind. The PER:mCRY complex then inhibits the CLK:BMAL1 complex (Zhu et al., 2006, Yuan et al., 2007). 4. The CLK:BMAL1 complex otherwise promotes the production of PER, TIM and mCRY and therefore completes the 1st core negative feedback loop with both complexes affected interacting with circadian clock regulated pathways (Darlington et al., 1998, Lee, Bae and Edery, 1999, Zhu et al., 2006, Yuan et al., 2007). 5. The CLK:BMAL1 complex also promotes PDP1 and VRI in a secondary negative feedback loop, however both act on CLK with PDP1 being a promoter while VRI is an inhibiter. An additional protein is produced (CWO) that also inhibits it's own promoter, creating another negative feedback against CLK:BMAL1 function (Cyran et al., 2003, Kadener et al., 2007, Richier et al., 2008, Zhan et al., 2011, Derks et al., 2015.). 6. A photoreceptive dCRY is activated by light and interacts with TIM to trigger degradation, also recruiting JET for such, therefore inhibiting formation of the PER:TIM:mCRY complex (Emery et al., 1998, Zhan et al., 2011, Derks et al., 2015, Berntsson et al., 2019). It is unclear how much of this molecular architecture is applicable to *P. xylostella,* however many of the clock genes detailed here have been found in numerous moth species, including some in *P. xylostella* directly (Kontogiannatos, Gkouvitsas and Kourti, 2016).

The circadian clock mechanism of *D. plexippus* has similarities to findings from both *D. melanogaster* and *Mus musculus* models. The genes *per* and *tim* were not only the first two molecular components of this clock system discovered but also are still shown to make up a key cyclic part of the circadian clock model across animal life (Rosato, Tauber and Kyriacou, 2006). The proteins these genes produce, PER and TIM, bind together as a heterodimer complex that inhibits its own expression via interactions with another heterodimer, the CLK-CYC complex. Both these complexes form the core TTFL, relying on a system of negative and positive feedback loops. With *D. melanogaster*, TIM in these models also interacts with a light sensitive pathway involving the use of CRY, a light receptive protein, to generate a light sensitive molecular system which allows light to

entrain the circadian clock (Zeng et al., 1996, Hardin, 2005). The Lepidopteran clock functions in a similar way, relying on light receptive dCRY proteins and TIM degradation pathways, however, *D. plexippus* was shown to also possess mCRY. mCRY takes the place of TIM, entering the nucleus with PER to repress their own expression while TIM still interacts with the light sensitive pathway but is lost from the complex after phosphorylating PER to allow for nucleus entry (Zhu et al., 2006, Yuan et al., 2007, Kyriacou, 2009). The levels of influence circadian mechanisms have has been studied in many organism with increasing focus on its importance at governing time specific toxicities (chronotoxicity) (Pszczolkowski, Dobrowolski and Spencer, 2014, Xie et al., 2019, Butt, Qayyum and Jones, 2020).

1.3.3.6 Lepidopteran circadian physiology

Circadian rhythms influence many aspects of lepidopteran and pest species physiology, including the immune system and anticipation of species interactions, helping to keep pests in time with both their hosts and threats (Kim et al., 2011, Scheiermann, Kunisaki and Frenette, 2013, Curtis et al., 2014). The two major clades of Lepidoptera are notably split via preferred active phase with moths being nocturnal and butterflys diurnal (Kawahara et al., 2018). The circadian clock's importance can be demonstrated by the variety of cells and organs within the body that express them, relying on 'well-timed' pathways to maximise efficiency and avoid disease (Chaix, Zarrinpar and Panda, 2016, Aoyama and Shibata, 2017, Serin and Acar-Tek, 2019). One such key site is in the gut where rhythmic feeding and the subsequent digestion and excretion of consumed foods and potential toxins can be observed. These peripheral circadian clocks are however mostly dependent on inputs from the central clock mechanism. One of the peripheral clocks most likely to become uncoupled from the CCN however are those involved in digestion due to the variety of environmental and endogenous signals they are exposed too (Reppert and Weaver, 2002, Albrecht, 2012). The lepidopteran midgut shows evidence of active

circadian clocks, including midgut light entrainment in decapitated larvae, potentially showing the importance of these peripheral clocks maintaining daily rhythmicity as they do so lacking CCN and sensory organs (Sauman and Reppert, 1998, Nobata et al., 2012). The mid gut peripheral clock was also shown to be important in the regulation of detoxification genes in addition to digestion (Zhang et al., 2021). The rhythmicity of detoxification genes may be crucial for anticipating the changing presence of secondary metabolites during plant herbivory. Research on the phloem feeding pest *B. tabaci* has shown it must outcompete the production of indolic glucosinolates in brassica hosts phloem sap which are upregulated via *cca1* pathways, expressed after dawn in plants (Green and Tobin, 2002, Lei et al., 2019, Manivannan et al., 2021). In addition to the circadian clock's importance for the regulation of organism's physiology, it is very sensitive to changes, showing varying ranges of entrainment capabilities and sensitivity to a large variety of environmental signals, potentially including plant hosts (Rieger et al., 2012, Zheng et al., 2021).

Specific daily behavioural rhythms with evidence for clock expression in both the head and peripheral tissues has been shown in Lepidoptera. Peripheral tissue clocks can react differently to CCN clocks depending on light regime changes (Iwai et al., 2006) and may not rely on inputs from CCN (Nobata et al., 2012). Core molecular clock components, such as PER and TIM, are still crucial for maintaining biological rhythms in peripheral clocks, shown in *B. mori* (Ikeda et al., 2019). Many aspects of lepidopteran biology are 'well-timed' with egg hatching showing morning anticipation as well as maintenance of rhythms in constant conditions (Minis and Pittendrigh, 1968, Sakamoto and Shimizu, 1994). Sexual activity shows strong circadian rhythmicity with high levels of variation between species, potentially facilitating speciation events (Groot, 2014). Temporal distinctions to avoid competition is known across many species (Popp, Ficken and Reinartz, 1985, Droge et al., 2016) and therefore may be used by lepidopterans during mating and or feeding. Studies

have previously shown that lepidopteran feeding and digestion can be rhythmic with larval growth and midgut gene expression showing circadian rhythmicity (Mohammadi et al., 2015, Suszczynska et al., 2017, Zhang et al., 2021), however, some species may avoid the use of circadian rhythms during feeding to adapt to both host defence strategies and the environment (Reynolds, Yeomans and Timmins, 1986, Niepoth et al., 2018). *P. xylostella* has been shown to express highly rhythmic oviposition rhythms, shown on *B. rapa,* where most egg laying took place within 2h from the beginning of dark phase, which also co-occurred with a peak in mating activity (Talekar et al., 1994). Circadian clock genes were also shown to be present in *P. xylostella* (Kontogiannatos, Gkouvitsas and Kourti, 2016), which therefore may be involved in the regulation of such rhythmic behaviours. Chronotoxicity to tochlorpyrifos was also found with the time between 10:00-16:00 of a 08:00-22:00 day night cycle being when *P. xylostella* was most susceptible, independent of the regulation of general detoxification pathways (Hou et al., 2010). Time of pupation was also significantly correlated with time of day with late afternoon to early morning having the highest levels of pupal emergence, with females pupating in the earlier part of this time frame (Gou and Qin, 2010).

1.3.4 Control of *P. xylostella* pest populations

1.3.4.1 Current pesticides used

40+ chemical pesticides are currently being implemented to control *P. xylostella* pest populations, making up the dominant form of *P. xylostella* pest management, while resistance levels to most pesticides continue to increase globally (Philips et al., 2014, Machekano, Mvumi and Nyamukondiwa, 2018). To help combat the increase in pesticide resistance gain in wild *P. xylostella* populations, pesticides such as chlorantraniliprole that select for increases in general detoxification pathway expression should be avoided, while deltamethrin and abamectin may be preferable as they elicit more minimal cross-resistance

responses (Liang, Gao and Zheng, 2003, Balasubramani, Sayyed and Crickmore, 2008, Kang et al., 2017). Spinosad is another pesticide that is currently being implemented that boasts a comparably low LC_{50} (Lethal concentration 50) with sub-lethal applications also significantly reducing *P. xylostella* population health and potential damage caused to crops (Yin et al., 2008, Abro et al., 2013), it may also be less toxic to non-target species and can increase the effectiveness of other pest management strategies (Liu et al., 2012). Along with spinosad, indoxacarb and emamectin benzoate have been shown to still be effective at controlling *P. xylostella* populations, though resistant populations of *P. xylostella* continue to arise (Hertlein et al., 2006). Interactions with host plants and other environmental factors may affect how successful pesticide treatments are at controlling *P. xylostella* populations (Abro et al., 2013), with interactions between different pesticides causing significant changes to efficacy. Mixing spinosad, emamectin and indoxacarb with bifenthrin increased their effectiveness compared to proportional levels of individual treatments (Attique, Khaliq and Sayyed, 2006). In the field however, farmers already use a mix of 3-4 pesticides to control *P. xylostella* (Mazlan and Mumford, 2005), therefore understanding and identifying synergistic effects may be important for reducing overall pesticide use for the effective control of *P. xylostella*, potentially also reducing the spread of resistances.

However, current pesticide control measures are susceptible to failure due *P. xylostella*'s increasing suit of chemical resistances (Shelton et al., 1993, Talekar and Shelton, 1993), creating a need for complex control strategies in response to *P. xylostella*'s seasonal invasions (Uthamasamy et al., 2011, Vitellie, 2018). The intensity of control measures and pesticide sprays currently employed can be taxing on small-scale farmers as well as having severe negative effects on the environment, often through the breakdown of ecosystem interactions and pollution of water. Use of pesticides to these extents can also lead to human poisoning (Wilson and Tisdell, 2000, Aydinalp and Porca, 2004, RCEP, 2005, Jepson et al., 2014, Mahmood et al., 2016). Such practices may also have the adverse

effect of increasing *P. xylostella*'s prevalence in frequently treated fields via the loss of natural enemies and ecosystem stability (Pimentel, 1961, Emden and William, 1974, Uthamasamy et al., 2011), which may too lead to increased human health risks (Gottdenker et al., 2014).

1.3.4.2 Natural chemical alternatives and IPM

Attempts at reducing the reliance on pesticide treatments to control *P. xylostella* have been made, often via the implementation of IPM strategies. IPM offers tools, regularly natural in origin, to more effectively control pest species while minimising broad spectrum pesticide use (Ehler, 2006, Birch, Begg and Squire, 2011, Pretty and Bharucha, 2015), however, the prohibitive complexity of treatment schedules can cause farmers to lean back on such with more limited success so far at controlling *P. xylostella* (Sarfraz, Keddie and Dosdall, 2007, Norton et al., 2008). The use of *Bt*T has been widely used to compliment both pesticide treatments and IPM strategies (Navon, 2000, Schuler et al. 2003, Navik et al. 2019), however, after its application against *P. xylostella* there was rapid resistance gain, becoming the first pest to become resistant to *Bt*T (Tabashnik et al., 1994, Liu et al., 2020). Non-*Bt*T selected for wild strains of *P. xylostella* can still be susceptible however (Sarfraz, Keddie and Dosdall, 2007). Further targeted chemical treatments can also prove effective such as cuminaldehyde, which while showing high toxicity to multiple *P. xylostella* life stages, has more minimal effects to local species (Cai et al., 2020). Similarly useful 'non-harmful' chemicals that are being used are *P. xylostella* sex pheromones. The sex pheromones released by *P. xylostella* have been isolated (Lee, Lee and Boo 2005) and the use of these pheromones on *B. oleracea* show reductions in *P. xylostella* mating frequency and subsequently reduced larval populations, therefore theoretically reducing crop damage in the field (McLaughlin, Mitchell and Kirsch, 1994). These types of pheromones have also been used to bait *P. xylostella* traps (Mottus et al., 1997, Mitchell, 2002), additionally, advancements in synthesis techniques and efficiency of *P. xylostella* mating disruption

(Chen, Fang and Zhang, 2007) is leading to the successful application of field sprays. Other lepidopteran species' sex pheromones may also be useful as *Spodoptera* sex pheromones were shown to inhibit *P. xylostella* flight behaviour (Wang et al., 2021). However, while effective and non-environmentally damaging, sex pheromones mostly remain prohibitively expensive (Baker, 2008).

1.3.4.3 Biological control agents

Natural enemies/biological control agents that specifically target *P. xylostella* have been found, in the highest frequencies in south Africa (Kfir, 1998) and south China (Liu et al., 2000). Currently over 135 parasitoid species for *P. xylostella* are known, with members from 5 genera being implemented fully, the most common and successful being that of the Ichneumonidae wasp genera *Diadromus* and *Diadegma*, where members of these genera often account for most parasitism in multiple regions (Sarfraz, Keddie and Dosdall, 2007). Research continues to show these wasp genera can be effective at reducing *P. xylostella* populations (Lohr et al., 2007), in addition to increasing suitability of further biological control agents as the environment changes (Gurr et al., 2018). Yet, the implementation of these natural enemies face the same drawbacks as other biological control agents, finding difficulties with clearance for ecosystem introduction, pest species population specificity and habitat suitability (Simberloff and Stiling, 1996, Azidah, Fitton and Quicke, 2000, Sarfraz, Keddie and Dosdall, 2007). Efficacies of successful biological control agents however can be increased using previously mentioned *Bt*T (Schuler et al., 2003, Navik et al., 2019), though it is unclear how the spread of *Bt*T resistance will impede this.

1.3.4.4 Self-limiting strains

Irradiated and other infertile pests can be released to impede wild pest populations, however, developments in genetics has also allowed for the implementation of gene drive technologies, being used for SIT through the harnessing of CRISPR-Cas9 gene editing

tools to engineer self-limiting strains (Ran et al., 2013, Dunn and Follett, 2017, Kandul et al., 2019, Aumann, Hacker and Schetelig, 2020, Xu et al., 2022). Sex-alternate splicing of the *doublesex* gene was exploited for more stable SIT in lepidopterans, creating a female-specific lethality strain of *P. xylostella* (Jin et al., 2013). This has been used, using tetracycline to rescue female larvae in lab rearing, to not only reduce pest populations but also potentially delay *Bt*T resistance (Harvey-Samuel et al., 2015). Field trials using the sterile line of *P. xylostella* (OX4319L) has confirmed attraction of wild *P. xylostella* and demonstrated field dispersal levels (Bolton et al. 2019). Further studies have demonstrated the effective application of this gene drive technology to combat wild *P.* xylostella pest populations, with results showing OX4319L males released were competitive and able to disperse, however more tests are needed (Shelton et al. 2020) in addition to overcoming pushback against these kinds of technologies (Brossard et al., 2018, Callaway, 2018, Fears and Meulen, 2018).

1.3.4.5 Cultural practices

Traditional practices can be used, such as more diverse growing conditions, to promote pest control in combination with the drying of land and removal of crop residues as mentioned previously, typically preventing *P. xylostella* reservoir populations from forming (Sayyed, Rizvi and Alvi, 2002). Growing brassicas during wet seasons when available has also been shown to reduce *P. xylostella* prevalence on crops (Lim, 1982). The use of irrigation systems using sprinklers also disrupts *P. xylostella* development (Talekar, Lee and Huang, 1986), with it being shown that high humidity can significantly reduce *P. xylostella* emergence (Guo and Qin et al., 2010). Intercropping reduces the effects of cultivation monocultures by adding diversity to increase total crop resilience (Risch, Andow and Altieri, 1983, Landis, Wratten and Gurr, 2000). Historically, tomatoes have been used as a successful brassica intercrop with a ~1:4 ratio, significantly reducing *P. xylostella* population on brassica fields (Buranday and Raros, 1975, Sivapragasam, Tee and

Ruwaida, 1982). Collard brassica trap crops, used to draw pest species away from target crops (Shelton and Badenes-Perez, 2006), have also been harnessed with significant success (Shelton and Nault, 2004) and, when used in conjunction with targeted pesticide, has reduced *P. xylostella* populations on cabbage by up to 90% (Srinivasan and Krishnamoorthy, 1992).

1.3.4.6 Dead-end trap crops

Plants used as 'dead end traps' for *P. xylostella* have a history of success at reducing pest populations and therefore have the potential to reduce target crop losses (Talekar and Shelton, 1993, Veromann et al., 2014, Reddy, 2017, Gyawali et al., 2021). Research on trap crops for use with *P. xylostella* show various *Brassica* species being successful at reducing damage on target crops, however field applications were found to be less successful (Shelton and Nault, 2004, George, Collier and Port, 2009). *Barbarea vulgaris* (Rocketcress) was found to be a more effective alternative to *Brassica* species, with a 66x increase in oviposition compared to target crops in addition to very low *P. xylostella* larvae survival (Shelton and Nault, 2004, Badenes-Perez et al., 2014a). *B. vulgaris* is an effective trap crop due to both high glucosinolate content, which increases oviposition attraction with *P. xylostella*, and high triterpenoid saponin content, which is toxic to *P. xylostella* (Hussain et al., 2019), leading to the low survivorship shown. The efficacy of *B. vulgaris* as a trap crop can also be increased using *Bt*T producing transgenic strains (Shelton et al., 2008) and increasing soil sulphur content (Badenes-Perez, Reichelt and Heckel, 2010). The use of glucosinolate as an oviposition attractant can also be used to create dead end trap crops alongside target crops without the need for *B. vulgaris* co-cultivation (Zhu et al., 2020) or transgenic dead-end trap crops, as produced in *Nicotiana tabacum* (Cultivated tobacco) (Moldrup et al., 2012).

1.3.5 Harnessing circadian rhythms to improve the control of *P. xylostella*

Challenges associated with controlling *P. xylostella* are likely to be exaggerated by climate change, as atmospheric temperatures increase faster than predicted (Rahmstorf et al. 2007), also facilitating the expansion of *P. xylostella*'s core and seasonal ranges to include higher latitudes and elevations, furthering damage and costs. The increases in temperature will reduce *P. xylostella* egg production and development times (Liu, Chen and Zalucki, 2002, Bahar, Soroka and Dosdall, 2012), shortening the period available for farmers to respond to invasions, all while food sources are under increasing strain and risks (Parry, Rosenzweig and Livermore, 2005). In light of this and many aforementioned factors increasing the damage *P. xylostella* can cause, further novel research pathways to compliment control strategies should be investigated, with the study of chronobiology and circadian rhythms offering a possible avenue towards improved control methods, increasing the efficacy of current IPM strategies (Shirasu-Hiza et al., 2007, Goodspeed et al., 2012, Jander, 2012, Butt, Qayyum and Jones, 2020, Brady et al., 2021, Zhang et al., 2021).

Understanding of pest circadian rhythms can be integrated into IPM strategies for the targeting of treatments, such as chemical pesticide sprays, pheromones and SIT releases, to increase efficacy of treatments. Examination of time-dependent regulation and responses and the role they may play in treatment efficiency can be known as chronotoxicity (Pszczolkowski, 2008). Considerations of such are increasingly being studied for the enhancement of approaches and treatments in pest management (Blumenthal et al., 2001, Beauchamp and Labreque, 2007, Khyati, Malik and Seth, 2017, Zhang et al., 2021, Brady et al., 2021). Uncovering circadian rhythms in feeding behaviour will likely aid with understanding treatment susceptibility due to the clear rhythmic cycles associated with detoxification genes and produced enzymes pests use during herbivory and for pesticide resistance (Bagheri et al., 2016, Zhang et al., 2021). Feeding rhythms likely also dictate the

level of exposure to treatments pest species such as *P. xylostella* may have, which could be exploited to increase treatment efficacy and thus cost effectiveness (Zhang et al., 2021), helping reduce the volume of pesticides used on fields. The wide variety of other behavioural and physiological rhythms pest species may express, once known, can too be exploited such as *P. xylostella* oviposition and mating rhythms (Pivnick et al., 1990), aiding in the use of other IPM strategies such as natural enemies and pheromone sensitivity for mating disruption, including through SIT. Findings on physiological rhythms may also provide the ability to estimate chronotoxicity and effectiveness of certain treatments at differing times of day, increasing the usefulness of species-specific circadian rhythm studies (Hamby et al., 2013). Interactions with host plants have also been shown to effect pest responses to treatments and *P. xylostella* physiology (Campos, Schoereder and Sperber, 2004, Abro et al., 2013). In addition, shifting circadian clock entrainment between pests such as *T. ni* and brassica host plants has been shown to alter feeding significantly (Goodspeed et al., 2012), demonstrating that both pest and host plant circadian rhythms have significant roles in determining herbivory and therefore may also further affect responses to IPM treatments.

1.4 Conclusions and project aims

1.4.1 Conclusions of review of literature

It is evident through research into the effects of circadian rhythms on physiology and chronotoxicity that perhaps the biggest hurdle to overcome in its application to pest management is the complexity and species-specific nature of interactions between circadian clocks, behaviours and the natural environment. However, both the research and technology needed to uncover these interactions is increasing, along with the need for such due to increasing awareness of the damage the overuse of pesticides is causing (Aktar, Sengupta and Chowdhury, 2009, Gill and Garg, 2014, Gunstone et al., 2021). Climate

change will compound these problems also, as pests increase in prevalence and developmental speed in ecological unstable environments cleared of natural enemies and or competition (Uthamasamy et al. 2011, Hill, Macfadyn and Nash, 2017, Deutsh et al., 2018, Savary et al., 2019, Skendzic et al., 2021), while food scarcity and starvation become an increasing threat (Ericksen, Ingram and Liverman, 2009, Cribb, 2010). However, the awareness of how chronobiology and chronotoxicity affect species' physiology and pest management has been increasing while barriers to understanding how such may function or be implemented (Pszczolkowski, 2008, Pszczolkowski, Dobrowolski, Spencer, 2014) are decreasing. The uptake of IPM treatments that rely on species-specific physiology and interactions, including with natural or engineered plant defence pathways, will continue to provide further opportunities for circadian clocks and rhythms to be harnessed to improve the efficacy of such. The previously discussed (in: 1.2.4.1, 1.2.5) technological advancements are allowing more detailed and deeper understanding of the physiological networks at play and how they may be regulated by the circadian clock, with high-throughput integrated omics making such research cheaper and faster (Misra et al., 2019). These advancements will make the application of circadian rhythms to IPM increasingly achievable and assist the transformation towards precision agricultural techniques (Strickland, Ess and Parsons, 1998, Natikar, Balikai and Anusha, 2016, Barnes et al., 2019), improving both the economic and environmental outcomes of pest management.

It's clear that *P. xylostella* is, and will continue to be, one of the most damaging and costly lepidopteran pests in the world, at least for the foreseeable future. Though many measures are being taken to improve chemical pesticide treatments, due to both the life history traits of *P. xylostella* and its ability to rapidly gain pesticide resistances discussed previously (in: 1.3.3.3, 1.3.3.4), such treatments will likely prove unsustainable in the long term. Continuing research shown previously (in 1.3.2.4) into the origin of *P. xylostella* is proving fruitful with further possible natural enemies being found, however, the precise origin

region is still highly debatable with evidence for 3 widely distant regions, though all within ~30° latitude of the equator. IPM treatments are being implemented and tested with the use of synthesised *P. xylostella* sex pheromones and SIT releases providing potentially species-specific pest population control (McLaughlin, Mitchell and Kirsch, 1994, Chen, Fang and Zhang, 2007 Jin et al., 2013 Bolton et al. 2019 Shelton et al. 2020 Wang et al., 2021). However, as both climate change and an incomplete evolutionary host range expansion threaten to increase the scale of *P. xylostella* damage (Sayyed, Rizvi and Alvi, 2002, Knolhoff and Heckel, 2011, Henniges-Janssen et al., 2011a,b), it is apparent that synergistic combinations of both modern and traditional control practises should be considered. Harnessing circadian rhythms offers another possible avenue to increase the efficacy of treatments, exploiting rhythmic physiology found in *P. xylostella* and other lepidopteran pests. These rhythms, which can also be found in *P. xylostella* host plants and their defences, discussed previously (in 1.2.2.6), have been shown to significantly affect treatment efficacy and herbivory (Abro et al., 2013, Goodspeed et al., 2013a,b). Consequently, species-specific physiological rhythms, once known, may be harnessed to increase IPM efficacy and therefore uptake by reducing costs, subsequently leading to reduced environmental damage from pesticides currently used to attempt to control *P. xylostella*.

1.4.2 Research project aims

Possible avenues to improve *P. xylostella* pest management through chronobiology have become evident, shown previously (in: 1.2.6, 1.3.5), including optimising the timing of control measures and the manipulation of relevant daily timekeeping in plants and or pests. To pursue this, the study of species-specific physiological rhythms and the separate and combined impact of associated abiotic and biotic factors will be required. Control of *P. xylostella* may be enhanced by exploiting their physiological rhythms in IPM strategies, as

they show rhythmic reproductive behaviours and pheromone sensitivity, and at least some molecular interactions with host plants have been characterised through studies discussed previously (in 1.3.3). Similar sensitivities have also been shown (in: 1.3.2, 1.3.3) to include plant volatiles and therefore interactions with such produced by host plants may inform *P. xylostella* physiological rhythms. Research has shown (in 1.2.2.6) that interactions between pest and plant host circadian clocks can also inform herbivory resistance in brassica plants, so, understanding how *P. xylostella* interacts with brassicas as a specialist feeding insect herbivore may too be important for crop protection. Considering both the findings and gaps in the literature reviewed in this chapter, *P. xylostella* behavioural and molecular rhythms were investigated, along with how such interact with host plant circadian clocks and defence pathways. Therefore, potentially providing important information for the enhancement of *P. xylostella* IPM strategies via the precise timing of pest management treatments with uncovered *P. xylostella* physiological rhythms and interactions with natural plant defence pathways. Consequently, the main aim of this project was to demonstrate the locomotor and feeding rhythms of *P. xylostella* and what potential mechanism may be controlling such, addressing this through two aims:

- **How is adult *P. xylostella* behaviour controlled and expressed across the 24h day? (Chapter 3).** Adult *P. xylostella* moths mediate population spread through reproductive and migration behaviours. To gain insight into the factors governing adult rhythmic behaviour, the impact of light, temperature and the circadian clock on adult locomotor behaviour of individual moth and on near-food activity of group-housed moths was determined. Differing environmental cycles of light and/or temperature were used for this purpose. In addition, molecular circadian rhythms of key circadian clock genes were monitored in adult *P. xylostella*. The behavioural rhythms and relative importance of light, termperature and the circadian clock for such in *P. xylostella* adults is presented in Chapter 3. These

findings may be applicable to improve the efficacy of relevant pest management strategies that impact adult *P. xylostella* pest populations such as through the timing of pesticide and pheromone sprays, natural enemies and SIT release and potentially also interference with migratory responses and host plant attraction.

- **How is *P. xylostella* larval herbivory controlled and expressed across the 24h day? (Chapter 4).** *P. xylostella* larval feeding directly damages agricultural crops. Diel rhythms in feeding likely reflect the combined influence of environmental and circadian rhythms in both *P. xylostella* larvae and host plants. The separate and combined impact of these factors were assessed in time course leaf tissue loss assays in which daily light cycles, host plant genotype and alignment of larval and leaf circadian phases were systematically varied. Additionally, daily transcript profiles were determined for both circadian clock genes and detoxification genes in *P. xylostella* larvae. Larval feeding rhythms and the relative importance of light and *P. xylostella* larvae and host plant circadian clocks are presented in Chapter 4. These findings may be applicable to improve the efficacy of relevant pest management strategies that impact larval *P. xylostella* pest populations such as through the timing of pesticide sprays when larvae show the highest activity and consumption or lowest levels of detoxification proteins. These rhythms may also demonstrate increased levels of exposure to possible natural enemies and chemical toxins, potentially also being useful for the improved timing of natural plant defences to outcompete *P. xylostella*.

Chapter 2 Methods and Materials

2.1 Culture maintenance

2.1.1 *Brassica rapa* culturing

Brassica rapa seeds, variety `Wong bok' (Chinese cabbage), were supplied from E. W. King & Co Ltd and stored at room temperature until use. Single seeds were sown inside an inch-deep hole in the middle of 3 x 3 plastic square pots containing 'Levington F2 + sand' growing soil. Seeds were planted every week for a constant supply of food for *Plutella xylostella* culture maintenance. Plastic covers were used to maintain moisture for a week after sowing of seeds before being removed. The plants were watered once every other day by an automatic flooding bench system in a temperature and light controlled roof-top glass house at Building 85, University of Southampton (UoS), maintaining ≥20°C day/ ≥18°C night. Supplementary lights were provided 07:00-19:00, to maintain at least 12 hours (h) of light during winter months. Plants were also maintained in environmental control rooms (ECR) in the insectary at certain conditions to be entrained with the environmental conditions larval *P. xylostella* are exposed to for experiments or in OP with such, before being used in for experiments or as feed.

2.1.2 *Arabidopsis thaliana* culturing

Wild type (WT) Col-0 *Arabidopsis thaliana* were grown as plant feed for *P. xylostella* larvae feeding experiments, then as feed for entrainment of larvae before being fed on mutant *A. thaliana* strains. WT *A. thaliana* was grown in the UoS glasshouse maintaining ≥20°C day/ ≥18°C night, lights on 07:00-19:00, watered every other day via flooding bench. *A. thaliana* was then entrained to conditions the larvae were kept in before carrying out experiments. Plants were also grown in an ECR for seed bulking and collection, where they were maintained at 60%RH, 23°C, 16/8 Light/Dark (L/D) cycle and watered by hand

once a week. Seeds were sown over the top of the soil made 1:1:1 of Levington F2 + sand growing soil, John Innes number 3 and Vermiculite. The soil was also treated with Imidasect pesticide for seed bulking only. As seeds germinate, seedlings were removed until only 2 plants remain per pot before encasing the plants in a tall plastic cover. The seeds of JA mutant lines were given by Professors John Turner (Emeritus at University of East Anglia) and Ivo Feussner (University of Göttingen), while clock mutants were given by Professor Antony Dodd (John Innes Centre), shown in Table 2.1.

Table 2.1 Mutant *A. thaliana* genotypes

Showing mutant identifyer, full name of affected gene and associated pathway. References provided for first use and creation of mutants.

Mutant	Mutant full name	Affected pathway	References
aos	Allene oxide synthase	JA biosynthesis mutant – conversion of fatty acids to OPDAs	Park et al., 2002
opr3-1	Oxophytodienoate-reductase 3	JA biosynthesis mutant – reduction of 12-OPDA to OPC-8:0	Stintzi and browse, 2000
coi1-16	Coronatine-insensitive protein 1	JA signalling mutant – activated by JA-Ile to repress MYC	Ellis and Turner, 2002
CCA1-ox	CIRCADIAN CLOCK ASSOCIATED 1 – overexpressor	Core circadian clock mutant – Continuous promotion of *CCA1* expression	Wang and Tobin, 1998
cca1-11/lhy-21/toc1-21	CIRCADIAN CLOCK ASSOCIATED 1/LATE ELONGATED HYPOCOTYL/ TIMING OF CAB EXPRESSION 1	Core circadian clock mutant – loss of function of the 3 core plant circadian clock genes	Ding et al., 2007

coi1-16 (*coronatine-insensitive 1*) mutant plants are male sterile at ambient and rearing temperatures. This particular allele of *COI1* shows temperature sensitivity, therefore the plants were moved to a cooler ECR set to 16°C to allow for pollen development, fertilization and seed formation (Ellis and Turner, 2002). The mutants' *aos* (*allene oxide synthase*) and *opr3-1* (*oxophytodienoate-reductase 3*) are deficient in the production of

jasmonic acids that are required for pollen development, fertilization and seed formation. Therefore, flower buds of those lines were dipped in solution jasmonate solution (10mM) every 3 days to stimulate the production of seeds (Acosta and Farmer, 2010). Clock mutants *cca1-11/lhy-21/toc1-21* (*circadian clock associated 1-11/late elongated hypocotyl-21/timing of CAB expression 1-21*) (TM) and *cca1-ox* (circadian clock associated 1–overexpressor) were grown in the same conditions as WT *A. thaliana* in ECR.

2.1.3 *P. xylostella* culturing

P. xylostella populations were reared within ECR at the UoS Insectary at 20°C in 12/12 L/D. W30xD30xH30cm BugDorms were used to house mixed sex *P. xylostella* populations. The population was acquired from Rothamsted Research, (ROTH strain wild collections during the 1960s) and has been continuously maintained on *B. rapa*. Adults were placed into BugDorm enclosures with a beaker of sugar-distilled (d) water (~25% table sugar) saturated cotton wool as an energy rich liquid food source for adult *P. xylostella* to feed on (Krenn, 2014). Two 4+ week-old *B. rapa* plants with minimal pest damage and age-related deterioration were collected from glasshouse growing set up at UoS and were placed in water dishes in the enclosure. Plants were watered by hand to prevent wilting or flooding, ensuring plants were suitable for *P. xylostella* egg laying while protecting such from drowning. After *P. xylostella* eggs appear on plant foliage the exposed plants were transferred into a fresh BugDorm with more fresh plants in the same setup as before. Once eggs hatched, evident by 1st instar larvae leaf mining, additional fresh plants were moved into the enclosure to provide a constant food supply as herbivory took place. Larvae either pupated on their own or were induced to do so by the exhaustion of food supply, with the latter resulting in smaller adults so is avoided for experiments (Helm et al., 2017). Once pupae appear, a fresh adult food beaker was placed within the enclosure, to provide food for any adults that eclose. Eclosed adults were moved using a

mechanical insect aspirator (MECHANICAL POOTER by Watkins & Doncaster) to a fresh BugDorm enclosure with the same setup noted, with adults chosen at random and mixed with offspring from other enclosures to reduce consecutive inbreeding.

Artificial diet made using a recipe based on a number of papers was also trialed to provide an arrythmic food source for both *P. xylostella* maintenance and experiments (Shelton et al., 1991, Guanghong et al., 1996, Carpenter and Bloem, 2002 and Collins et al., 2010). The substrate from the recipe trialed allowed for *P. xylostella* egg laying and hatching, however, 1st intsar larvae didn't survive, with no transferred later instar larvae recorded gaining weight from the artificial food source.

2.2 Locomotor activity assays

2.2.1 *P. xylostella* locomotor assay system

In preliminary activity monitor experiments, three sugar substrates were trialled in different media, cotton wool and agar. Cotton wool saturated with sugar water, like adult *P. xylostella* were fed during population culturing, diluted honey saturated cotton wool and the selected sugar-agar mix substrate. Both sugar water and diluted honey saturated cotton wool had a high number of moths becoming trapped by the cotton, along with humidity within bunged tubes appearing high due to apparent condensation or cotton wool run off. Both these issues with *P. xylostella* becoming trapped and condensation problems led to the selection of the sugar-agar mix for use in activity assays, this to preserve the activity and accuracy of *P. xylostella* activity counts during the adult *P. xylostella* assays.

Individual *P. xylostella* activity counts were detected by TriKinetics LAM25 assay system using 25 mm diameter glass test tubes. When an infra-red beam at the midpoint of the tube was disrupted by the moth moving past, this was detected as a count. Each tube was filled with energy-rich sugar-agar substrate (1 % agar, 5 % table sugar, 0.07% Methyl 4-

hydroxybenzoate) at the bottom 2 cm and a cotton plug at the top to allow gas exchange. Briefly, the food substrate was made by mixing d water, sugar, and agar to a boil then, bringing the temperature down to 60°C to add preservative Methyl 4-hydroxybenzoate from an ethanol solution.

2.2.2 *P. xylostella* locomotor assay experimental conditions

In preliminary experiments using a constant temperature of 23°C in experimental setups, >50% of moths would cease activity before at least 6 days of data could be collected. This issue was partially fixed by reducing the temperature of standard conditions to 17°C.

The TriKinetics assay system using LAM25 was kept inside a black plastic tub at 17°C with local humidity provided by a tray of algaecide and fungicide treated d water. White LEDs (light emitting diode) were fixed inside the black plastic tub providing 8µmol/m^2/s (~400 lux) of light at 12/12, 6/6/6/6, 14/10, 16/8, 18/6 and 20/4 h of L/D light cycles or at constant light (L/L) or constant dark (D/D), shown in Table 2.2. For each locomotor assay, 16 male and 16 female *P. xylostella* were selected from culturing enclosures kept at 12/12, 20°C and were recorded over 6+ full days. The data was collected by a DAM (Drosophila activity monitor) system (Pfeiffenberger et al., 2010) in 5 min segments (bins) and was subsequently exported to ClockLab Analysis Version 6 (ActiMetrics, 2019) for analysis.

Table 2.2 Conditions maintained over 24h during locomotor activity assays.

Light cycle conditions denoted as hours (h) of light/ h of dark. Constant dark as D/D and constant light as L/L. Dawn refers to period of increasing light intensity, dusk refers to period of decreasing light intensity with rhythms of abiotic factors for April and June shown in Fig. 2.2.

Condition	Light cycle	Temperature
D/D	Constant Dark	17°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

A population of 30 adult male and 30 adult female *P. xylostella* moths were also analysed in a BugDorm cage using an DPM (Drosophila population monitor), shown in Fig. 2.1. This allowed for adult rhythms in activity proximal to a food source to be measured in a system in which a mixed-sex population of moths could fly freely and interact with each other. This system was set up in the same environment as stated for the previous method, however using the sugar source provided in stock cages for adult *P. xylostella* rearing.

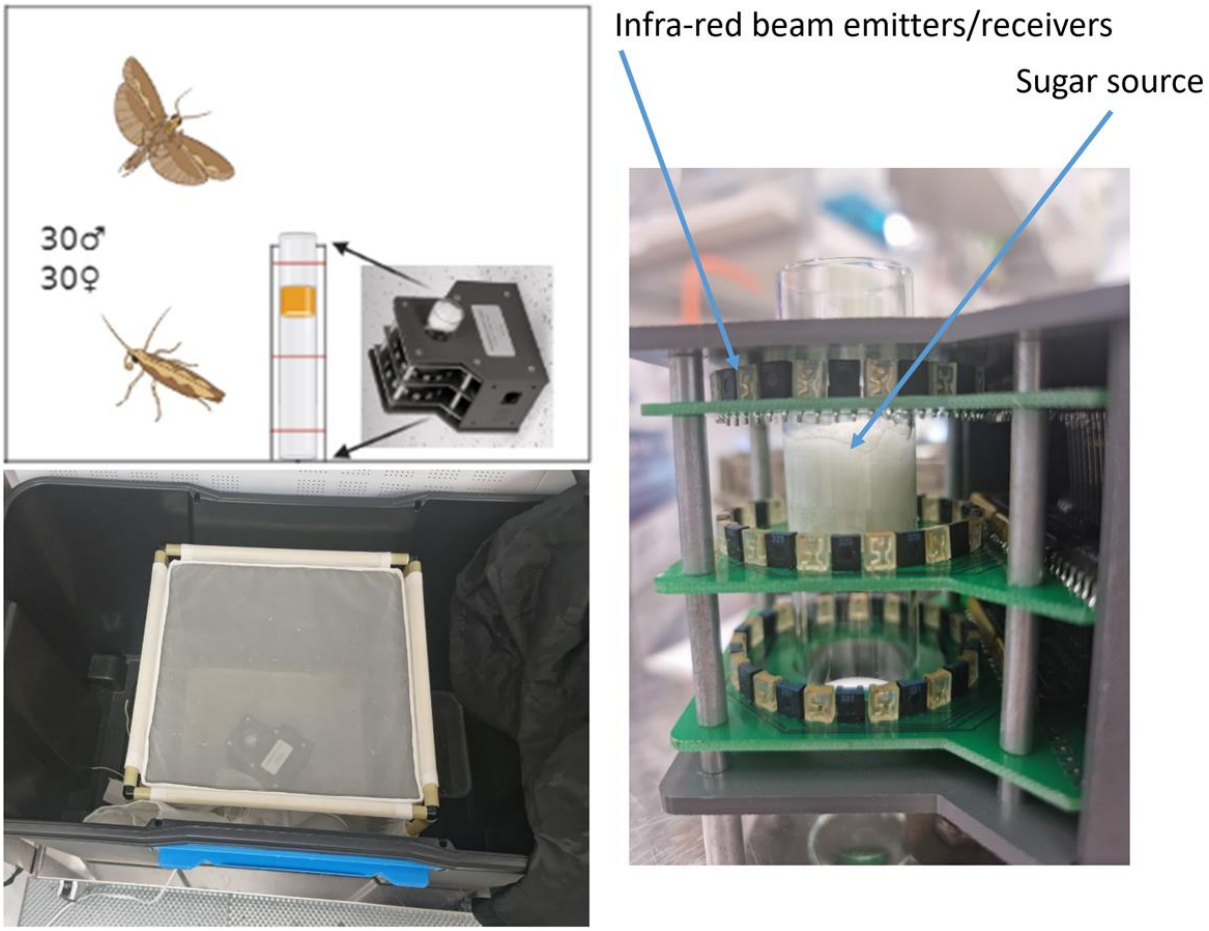


Figure 2.1 Group population assay set up.

Set up with 30 male and 30 female moths inside BugDorm cage with DPM system so that only the first infra-red beam is exposed. Orange plug on diagram demonstrates where food source was located as shown on right image. Whole cage was placed into black tubs and fitted either with or without LED lights used in light cycle locomotor assays.

Environmental cycles mimicking light and temperature recordings in Kent, UK in April and June, were set up in a Percival incubator (model number: DR-36VL) based on environmental data from Shaw et al. 2019, producing the conditions shown in Table 2.2. The recorded environmental profile in the incubator setups is shown in Fig. 2.2 where an LED lamp was used to create ramping dawn and dusk light with the incubator maintaining the temperature profile, with max light producing 38µmol/m^2/s (~2000 lux). LAM25 monitors were used in these conditions following the same assay protocol as above.

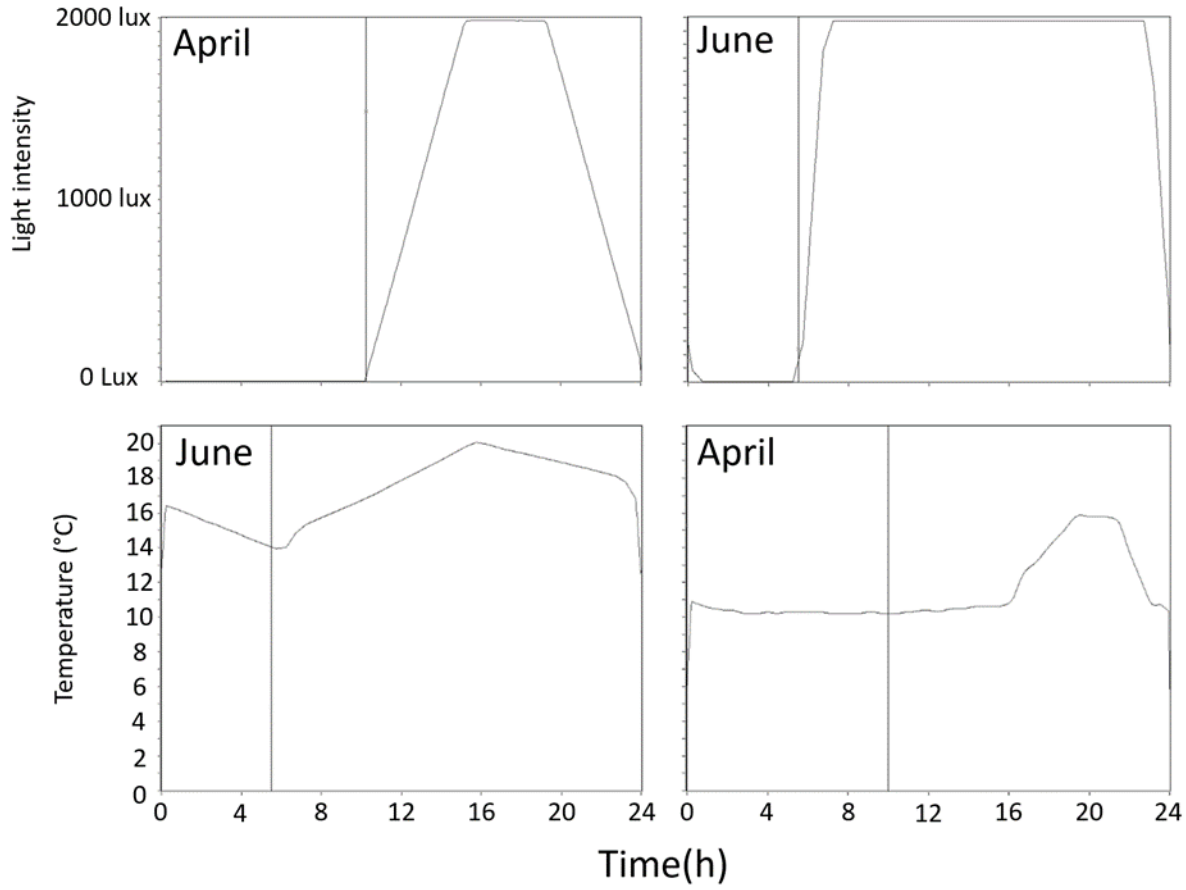


Figure 2.2 Semi-field incubator conditions.

June and April mimic light and temperature profiles as recorded by a DEnM (Drosophila Environment Monitor by TriKinetics) inside Percival incubators set using environmental data from Shaw et al. 2019. Drop offs in temperature near beginning and end of temperature profiles is an artifact of analysis software presentation averaging data from environmental monitor across multiple days.

2.2.3 ClockLab locomotor activity analysis

ClockLab Analysis Version 6 software was used to analyse and produce graphical representations of *P. xylostella* locomotor activity data (activity counts) and to quantify specific parameters of such through activity profiles and periodograms of both individual moths and mean average of samples. Double plotted actograms that show 1st day normalized against 2nd day along first row of graph, 2nd against 3rd on second row and so on, was used to show average between days in 30-minute bins of counts. The day at which individual moths stopped producing data was recorded as point of death for survival in

conditions between male and females. 6 day averages were used to produce activity profiles for individual moths and then used to collect quantitative data. Average dark phase and light phase activity (including s phases in constant conditions) was recorded using the software from these 6 day activity profiles, combining the two to record total activity across the 24h day. The dark and light phase activity was then divided by the number of h of dark in each condition to show phase activity rate. The initial response to dark phase onset was shown using activity counts recorded within 2h of such. Periodograms produced using the software show the significant likelihood of specific rhythmic periods in data and was used to determine amplitude and time duration of rhythmic periods of individual moths with significance threshold set to $P = 0.01$. Relative rhythmic power (RRP) was then calculated using the ratio between the periodogram's dominant period likelihood amplitude and significance threshold for h length of specified period.

2.3 Feeding assays

2.3.1 Leaf area loss image analysis

P. xylostella larvae in instars 3+ were used for larval feeding experiments. They were identified by colour and size (Moriuti, 1986). The larvae were collected using two paintbrushes to coerce a larva into attaching a silk thread to a brush then moving to the wanted position before disconnecting the thread, attempting to minimize handling effects.

Prior to use*, P. xylostella*, *B. rapa* and *A. thaliana* cultures were entrained to the conditions of the experiment they would be used in using UoS insectary ECR to maintain constant conditions of 20°C and appropriate light cycles with lights on intensity of 40µmol/m^2/s ~2300 lux. Image data was collected using the time-lapse function of a Bushnell 24mp trophy cam HD. The time-lapse was set to run continuously taking an image every 30-minutes. The camera was positioned above a W30xD30xH30cm BugDorm with a side panel entry port facing upwards, so the bottom panel was visible. The 'healthiest' 3rd

rosette leaves that were slightly smaller than the dishes (1 per dish/repeat) were cut from *B. rapa* plants at the time of experiment set up. *A. thaliana* 3+ rosette leaves were collected (16 per dish/repeat) before the plants bolted as this may significantly alter leaf nutritional value and volatiles (Hinckley and Brusslan, 2020). To prevent leaf desiccation a piece of d water saturated cotton wool was wrapped around the stem of used *B. rapa* leaves and *A. thaliana* leaf stems were threaded into micro-Eppendorfs filled with d water before being used in setups. 5 larvae of the appropriate instars were chosen from the prepared *P. xylostella* culture randomly from provided entrainment food sources and placed into each dish. Before the dishes were placed into the BugDorm, a purple backing card piece was laid across the bottom, this provided contrast between background and leaves during image analysis. The set-up was then moved into a Percival incubator (model number: DR-36VL) 20°C, 70% relative humidity (RH) and appropriate light cycles with lights on intensity of 48µmol/m2/s (~2700 lux) for experiment recording. Covers for dishes were trialled but caused condensation to form, interfering with image collection. The elevated humidity may also have affected *P. xylostella* larvae (Lim, 1982, Talekar, Lee and Huang, 1986, Guo and Qin et al., 2010).

The light cycle conditions set were 12/12 L/D and L/L with differing levels of pre-exposure, demonstrating responses in extended circadian time (CT) compared to those where light schedules can inform circadian rhythms and clocks, also known as zeitgeber time (ZT). ZT time points start at 12 from dark phase onset, restarting at 0 from light phase onset. CT time points begin from light phase onset in L/L conditions, signifying the length in L/L without change in conditions. This entrainment schedule is shown in Table 2.3. *B. rapa* and *P. xylostella* cultures were also kept anti-phase to each other so when used together the larvae and leaf cuttings where in opposite entrainment, with the plants being the ‘time-travellers’. This also means in L/L experiments that plants are 12h deeper into constant conditions. To prevent plant tissue being exposed to opposite entrainment during

feeding, 12h recordings for OP L/D conditions were used so that OP plant material in their subjective (s) light phase was fed in the dark to *P. xylostella* larvae and then fresh OP plant material in their s dark phase was fed in the light to fresh larvae.

Table 2.3 Leaf area loss feeding assay entrainment and conditions.

Time course for *P. xylostella* larval feeding image assays. Lights on at 08:00, lights off at 20:00, moving into constant light for L/L experiments. 3 days of entrainment at 20°C in LD cycle prior to time course start denoted by condition labelling, L/L2 starts on plant and larvae's second dark phase loss and L/L3 on their third. Dark conditions shown by shaded cells. Assessments were done every 30 minutes.

Light
Dark

In-phase entrainment

Plant	08:00	20:00	08:00	20:00	08:00	20:00	08:00	20:00	08:00
Larvae	08:00	20:00	08:00	20:00	08:00	20:00	08:00	20:00	08:00
		12/12		L/L1		L/L2		L/L3	

Out-of-phase entrainment

Plant	08:00	20:00	08:00	20:00	08:00	20:00	08:00	20:00	08:00
Larvae	08:00	20:00	08:00	20:00	08:00	20:00	08:00	20:00	08:00
		12/12		L/L1		L/L2		L/L3	

After a >24h period of recording starting from (s) dark phase onset, the camera was collected, and recorded images downloaded. By moving between the images, the areas being eaten by *P. xylostella* larvae across each image frame were identified and tracked compared to changes in leaf shape due to leaf desiccation and or senescence after cutting. Using ImageJ software (Rueden et al., 2017), the area of leaf lost from feeding sites between each h was shaded. A colour pixel counting tool (Pichette, 2010) was then used to convert the amount of lost leaf area for each h into quantitative data for use in comparative stats and data presentation.

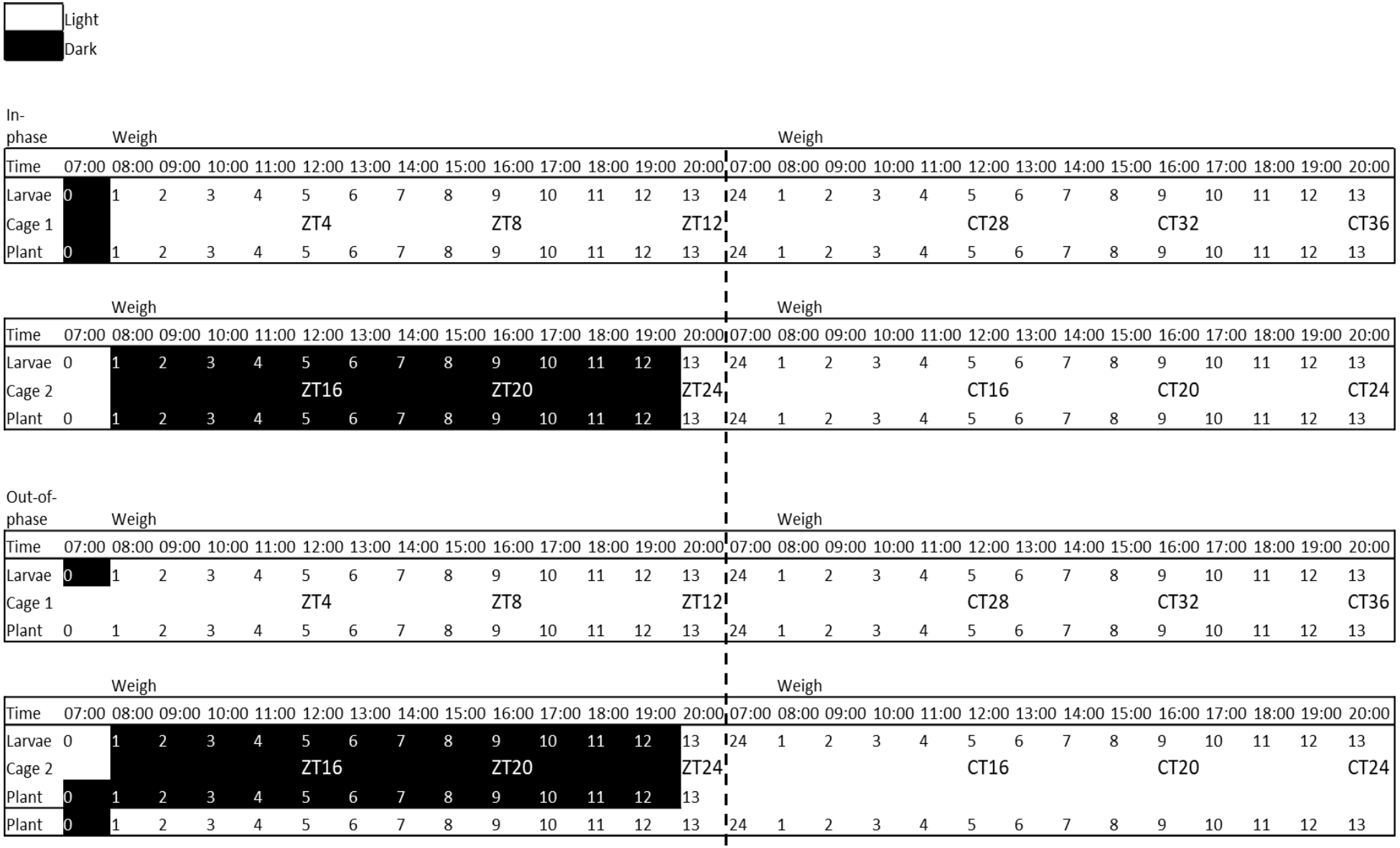
In addition to carrying out this assay on *cca1-ox*, TM, *aos*, *opr3-1* and *coi1-16* mutant *A. thaliana* leaves, this set up was repeated with equal number of differing *A. thaliana* genotype leaves on the top and bottom side of the dishes used as a feeding choice assay between WT and circadian clock mutant *A. thaliana*. Leaf area loss recording over the last two h of light phase and first 6h of dark phase of an L/D day of each *A. thaliana* genotype was recorded individually.

2.3.2 *P. xylostella* weight gain

The weight gain of *P. xylostella* larvae was measured after being fed on *B. rapa* leaves under different light cycle conditions and on OP leaves. Ten *P. xylostella* larvae per collection were collected under same protocol as previous feeding assay and then weighed using an Ohaus Analytical Plus at the start of each day before groups were placed into separate dishes with a *B. rapa* leaf and each left for certain amounts of time shown in Table 2.4.

Table 2.4 *P. xylostella* larval weight time course.

Time course for larval *P. xylostella* weighing schedule with lights on at 08:00 for Cage 1 and lights off at 20:00, with opposite cycle for Cage 2. 3 days of entrainment at 20°C in LD cycle prior to time course start where all larvae were weighed (10 for each time point) before being placed with leaves for each 24h day. Cage 1 starts constant light conditions past 20:00 and Cage 2 past 08:00. At collection points denoted by ZT (Zeitgeber Time) and CT (Circadian Time), larvae are weighed and returned to reserve stocks at the beginning of the h. Dark conditions shown by shaded cells. Number inside cell indicates what h of the 24h day it is, e.g. 08:00 to 09:00 is the 1st h, where weighing is done at the start of. Note the 10h time gap in the plot as indicated by the dashed line.


Light
Dark
In-phase
Weigh
Weigh
Time 07:00 08:00 09:00 10:00 11:00 12:00 13:00 14:00 15:00 16:00 17:00 18:00 19:00 20:00 07:00 08:00 09:00 10:00 11:00 12:00 13:00 14:00 15:00 16:00 17:00 18:00 19:00 20:00
Larvae 0 1 2 3 4 5 6 7 8 9 10 11 12 13 24 1 2 3 4 5 6 7 8 9 10 11 12 13
Cage 1 ZT4 ZT8 ZT12 CT28 CT32 CT36
Plant 0 1 2 3 4 5 6 7 8 9 10 11 12 13 24 1 2 3 4 5 6 7 8 9 10 11 12 13
Weigh
Weigh
Time 07:00 08:00 09:00 10:00 11:00 12:00 13:00 14:00 15:00 16:00 17:00 18:00 19:00 20:00 07:00 08:00 09:00 10:00 11:00 12:00 13:00 14:00 15:00 16:00 17:00 18:00 19:00 20:00
Larvae 0 1 2 3 4 5 6 7 8 9 10 11 12 13 24 1 2 3 4 5 6 7 8 9 10 11 12 13
Cage 2 ZT16 ZT20 ZT24 CT16 CT20 CT24
Plant 0 1 2 3 4 5 6 7 8 9 10 11 12 13 24 1 2 3 4 5 6 7 8 9 10 11 12 13
Out-of-phase
Weigh
Weigh
Time 07:00 08:00 09:00 10:00 11:00 12:00 13:00 14:00 15:00 16:00 17:00 18:00 19:00 20:00 07:00 08:00 09:00 10:00 11:00 12:00 13:00 14:00 15:00 16:00 17:00 18:00 19:00 20:00
Larvae 0 1 2 3 4 5 6 7 8 9 10 11 12 13 24 1 2 3 4 5 6 7 8 9 10 11 12 13
Cage 1 ZT4 ZT8 ZT12 CT28 CT32 CT36
Plant 0 1 2 3 4 5 6 7 8 9 10 11 12 13 24 1 2 3 4 5 6 7 8 9 10 11 12 13
Weigh
Weigh
Time 07:00 08:00 09:00 10:00 11:00 12:00 13:00 14:00 15:00 16:00 17:00 18:00 19:00 20:00 07:00 08:00 09:00 10:00 11:00 12:00 13:00 14:00 15:00 16:00 17:00 18:00 19:00 20:00
Larvae 0 1 2 3 4 5 6 7 8 9 10 11 12 13 24 1 2 3 4 5 6 7 8 9 10 11 12 13
Cage 2 ZT16 ZT20 ZT24 CT16 CT20 CT24
Plant 0 1 2 3 4 5 6 7 8 9 10 11 12 13
Plant 0 1 2 3 4 5 6 7 8 9 10 11 12 13 24 1 2 3 4 5 6 7 8 9 10 11 12 13

The average difference between proportions of gained weight to starting weight between each time point was used to work out weight gain at different time points over 24h. Average weight gain over time in the differing conditions was caleculated by minusing recorded proportional weight gain for previous time point in each set up. Two series is shown for cage 2 OP feeding due to plants used for L/L feeding time points remaining in light and so are 12h advanced into L/L conditions compared to used larvae, the same as for cage 1 larvae.

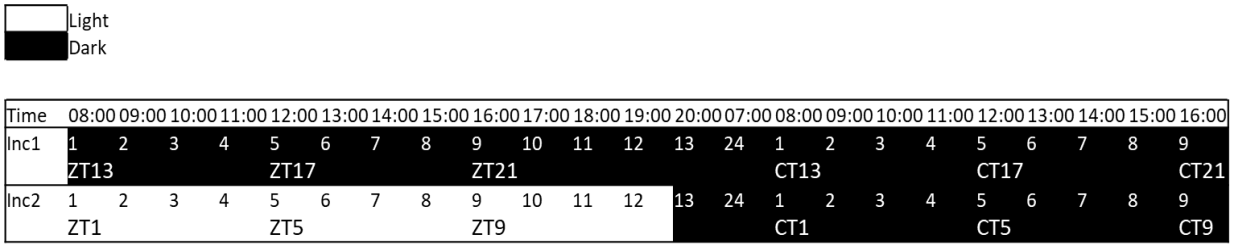
2.4 qPCR analysis

2.4.1 RNA collection and extraction

The sampling time course and entrainment light regime used to collect whole adult *P. xylostella* male moths is shown in Table 2.5. Briefly, only male moths were used as whole females are likely to carry eggs that can influence results depending on reproductive and oviposition rhythms (Tahoe, Mokhtarzadeh and Curtstinger, 2004).

Table 2.5 Time course for adult male *P. xylostella* collections.

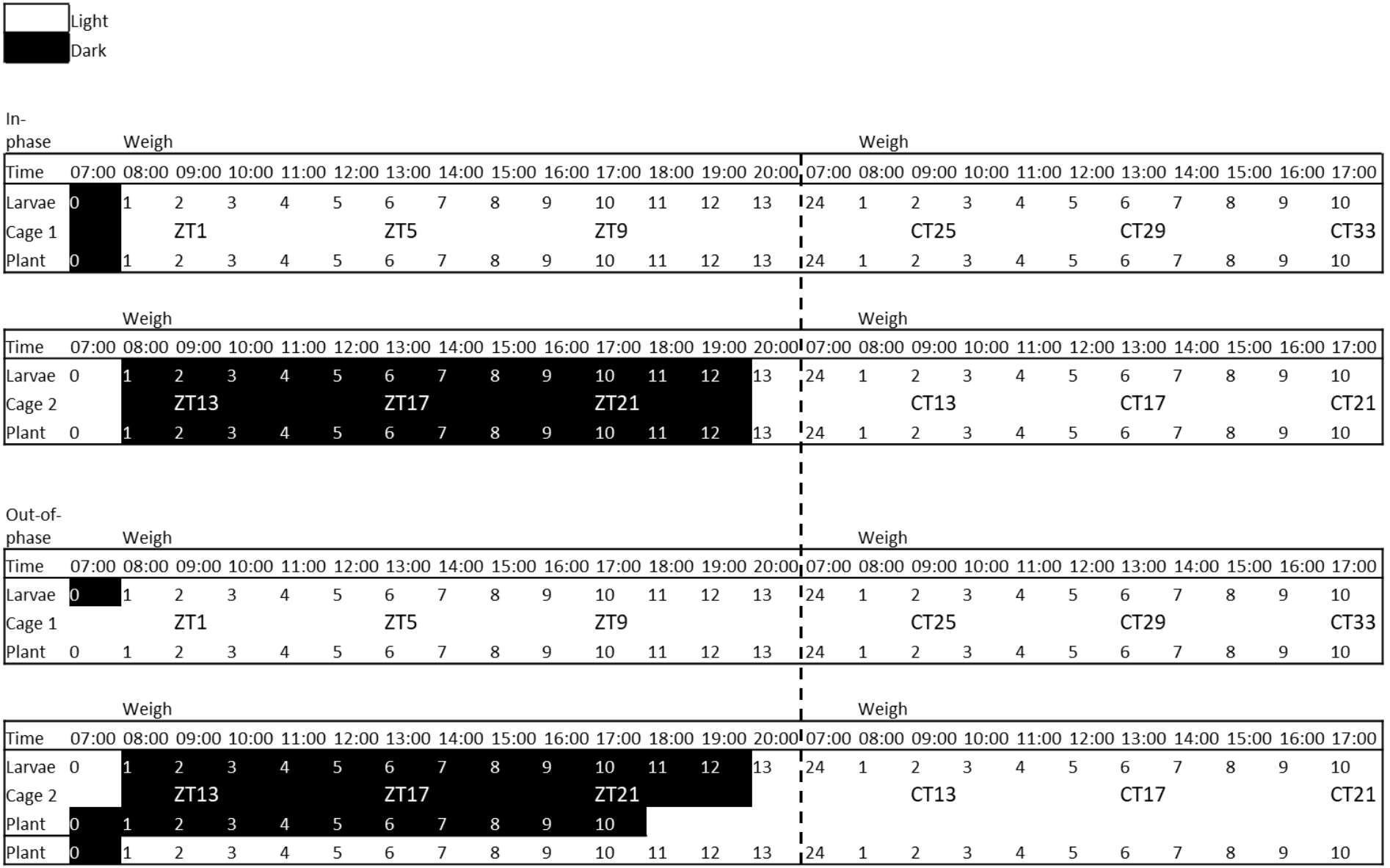
qPCR (Quantitative polymerase chain reaction) time course for adult male *P. xylostella* collections with lights off at 7 (07:00 on 24h clock) in Incubator (Inc) 1 and lights on at 19:00 with opposite cycle for Incubator 2. 3 days of entrainment at 20°C in LD cycle prior to time course start. Incubator 1 starts constant dark conditions past 19:00 and incubator 2 past 07:00. At collection points denoted by ZT and CT (ZT13 – 8:00 collection) 5 males are transferred into autoclaved Eppendorf tubes kept on dry ice before being transferred into -80°C long term storage. Dark conditions shown by shaded h bar.



The sampling time course and entrainment light regime used to collect whole larval *P. xylostella* is shown in Table 2.6. Larvae were removed from cages at random from in-phase (IP) leaves and OP leaves for their respective time courses. Male *P. xylostella* adults were collected via a mechanical insect aspirator, emptying the holding unit into dry ice frozen Eppendorfs using a funnel. The collected frozen adults were also doubled checked for correct sex before being moved to -80°C storage. The collected larvae were dropped directly into dry ice frozen Eppendorfs, then moved to -80°C for storage. These samples were used to extract RNA using RNAqueous-4PCR kit by Invitrogen (catalogue number: AM1914).

Table 2.6 Time course for larval *P. xylostella* collections.

Time course for larval *P. xylostella* collections for qPCR with lights on at 08:00 for Cage 1 and lights off at 20:00 with opposite cycle for Cage 2. 3 days of entrainment at 20°C in LD cycle prior to time course start, Cage 1 starts constant light conditions past 20:00 and Cage 2 past 08:00. Fresh plants are transferred to caterpillars 12h prior to time course start. Out-of-phase plant transfer and collection was done over two populations to avoid entrainment effects of LD phase. At collection points denoted by ZT and CT, 5 larvae are transferred into autoclaved Eppendorf tubes kept on dry ice before being transferred into -80°C long term storage at the beginning of the h. Dark conditions shown by shaded cells. Number inside cell indicates what h of the 24h day it is, e.g. 08:00 to 09:00 is the 1st h with ZT1 collection at 09:00. Note the 10h time gap in the plot as indicated by the dashed line.


Light
Dark
In-phase
Weigh
Weigh
Time 07:00 08:00 09:00 10:00 11:00 12:00 13:00 14:00 15:00 16:00 17:00 18:00 19:00 20:00 07:00 08:00 09:00 10:00 11:00 12:00 13:00 14:00 15:00 16:00 17:00
Larvae 0 1 2 3 4 5 6 7 8 9 10 11 12 13 24 1 2 3 4 5 6 7 8 9 10
Cage 1 ZT1 ZT5 ZT9 CT25 CT29 CT33
Plant 0 1 2 3 4 5 6 7 8 9 10 11 12 13 24 1 2 3 4 5 6 7 8 9 10
Weigh
Weigh
Time 07:00 08:00 09:00 10:00 11:00 12:00 13:00 14:00 15:00 16:00 17:00 18:00 19:00 20:00 07:00 08:00 09:00 10:00 11:00 12:00 13:00 14:00 15:00 16:00 17:00
Larvae 0 1 2 3 4 5 6 7 8 9 10 11 12 13 24 1 2 3 4 5 6 7 8 9 10
Cage 2 ZT13 ZT17 ZT21 CT13 CT17 CT21
Plant 0 1 2 3 4 5 6 7 8 9 10 11 12 13 24 1 2 3 4 5 6 7 8 9 10
Out-of-phase
Weigh
Weigh
Time 07:00 08:00 09:00 10:00 11:00 12:00 13:00 14:00 15:00 16:00 17:00 18:00 19:00 20:00 07:00 08:00 09:00 10:00 11:00 12:00 13:00 14:00 15:00 16:00 17:00
Larvae 0 1 2 3 4 5 6 7 8 9 10 11 12 13 24 1 2 3 4 5 6 7 8 9 10
Cage 1 ZT1 ZT5 ZT9 CT25 CT29 CT33
Plant 0 1 2 3 4 5 6 7 8 9 10 11 12 13 24 1 2 3 4 5 6 7 8 9 10
Weigh
Weigh
Time 07:00 08:00 09:00 10:00 11:00 12:00 13:00 14:00 15:00 16:00 17:00 18:00 19:00 20:00 07:00 08:00 09:00 10:00 11:00 12:00 13:00 14:00 15:00 16:00 17:00
Larvae 0 1 2 3 4 5 6 7 8 9 10 11 12 13 24 1 2 3 4 5 6 7 8 9 10
Cage 2 ZT13 ZT17 ZT21 CT13 CT17 CT21
Plant 0 1 2 3 4 5 6 7 8 9 10
Plant 0 1 2 3 4 5 6 7 8 9 10 11 12 13 24 1 2 3 4 5 6 7 8 9 10

Fresh plants were added 12h before time course start to ensure collected *P. xylostella* larvae were feeding on fresh leaves. This was particularly important for OP feeding due to possible effects of recent feeding on IP plant material. Two series were required for OP sample collection to avoid effects of entrainment from L/D phase transitions.

6 biological repeats were collected and after carrying out the RNAqueous-4PCR kit extraction protocol, isolated RNA was treated with DNase 1 also following the protocol provided by the RNAqueous-4PCR kit. The resulting RNA samples were tested for concentration and quality using a NanoDrop 1000 spectrophotometer. The results of which along with RNA concentration should show a A260/A280 ratio above 1.8, 2.0+ demonstrates high RNA purity with minimal contaminants from DNA or free nucleotides with A260/A230 results signifying presences of other residues (Matlock, 2015). Samples were aliquoted into an appropriate working volume (used for specific qPCR plate set ups) and were kept at -20°C for short term storage. This preserved the quality of RNA samples by reducing degradation effects of freeze-thaw actions, limiting such to 3 or less (Yu et al., 2017).

2.4.2 qPCR protocol

The RNA sample was used as template for one-step amplification by PrecisionPLUS OneStep qRT-PCR Master Mix manufactured by Primerdesign, with both a SYBR green florescent dye and ROX dye. qPCR was performed using an Applied Biosystems StepOnePlus Real-Time PCR System to quantify proportional levels of specific RNA transcripts between samples (Gibson, Heid and Williams, 1996). Amplification thresholds were used to calculate the proportional level of primer-specific transcripts present in each RNA sample using the $2^{-\Delta\Delta CT}$ method (Schmittgen and Livak, 2008). *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*) and glucosinolate sulfatase

genes *Plutella xylostella sulfatase 2* (*Sulf2*), 3 (*Sulf3*) and a primer pair that should amplify both at once (*Sulf2+3*), shown in Table 2.7. Primers were designed using Primer-3 and Primer-BLAST (Untergasser et al., 2007, Ye et al., 2012) to amplify desired distinct gene transcripts.

Table 2.7 Primers used for qPCR analysis.

Primer pair sequences and amplicon lengths for *P. xylostella* genes of interest used in qPCR experiments, adapted from Fu et al. 2013 and Ma et al. 2018, tested using qPCR serial dilution for efficiencies and 5% agarose gel electrophoresis to check expected amplicon lengths.

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%
Sulf2	5'-AGGACCCTTGCGAGCTGCGT-3'	5'-ACTTGGGGTCAGCGACGT-3'	129	108.0%
Sulf3	5'-CGGACCCTTGCGAGCTGCGA-3'	5'-CCCTGGGGTCAGCGGTGA-3'	129	102.4%
Sulf2+3	5'-GACTGGACCACCGAGGGTTA-3'	5'-AAGCGTCTTCGTTGCCGT-3'	136	93.0%

The qPCR machine used, (Applied Biosystems StepOnePlus Real-Time PCR System), enabled DNA amplification and reverse transcription of RNA within same solution using appropriate mastermix. PrecisionPlus OneStep qRT-PCR mastermix with both a SYBR

green florescent dye and ROX dye allowed for one step amplification. The ROX dye was used in addition to SYBR green to act as a fluorescence constant to control for differences across PCR plate recordings. Primer efficiencies were calculated using standard curve testing on a 10x dilution series from 100ng/µl to 0.001ng/µl and amplified amplicon length tested on 5% agarose gel against an ultra-low range DNA ladder (10-300 base pairs). A negative reverse transcriptase PrecisionPlus OneStep mastermix was also used as a control to check for contamination, as RNA should not be amplified. The well ratios used are shown in Table 2.8.

Table 2.8 qPCR plate well set-up.

Well ratios used for qPCR experiments. Ratios as described in PrimerDesign OneStep qRT-PCR mastermix protocol, with overall volume per well halved.

Component	Volume (µl)
PrecisionPlus OneStep™ qRT-PCR mastermix	5
Primer mix	0.5 (0.25 each)
Template RNA 1ng/µl	1
DEPC treated dH_2O	3.5
Total volume	10

The qPCR machine software produced the results as amplification thresholds and melt curve recordings. Amplification thresholds were used to calculate the relative expression of test gene transcripts present in each RNA sample using the $2^{-\Delta\Delta CT}$ method (Schmittgen and Livak, 2008). Briefly, the average amplification threshold of test and reference genes on a plate were used to determine the relative expression level using =Power(2,((Reference gene – Test gene) – (Reference gene time course mean – Test gene time course mean))). This method was used to determine relative expression levels of a target gene relative to a housekeeping gene as a reference, normalized against the differences between reference

and test gene overall gene expression levels on a plate. Melt curves were examined to confirm amplification of a single product of primers used and presence of possible contaminations

2.4.2.1 MIQE guidelines address

For the publication of results produced through qPCR, adherence to the minimum-information for publication of quantitative real-time PCR experiments (MIQE) guidelines is expected to ensure the reliability and reproducibility of such experiments (Bustin et al., 2009). Adherence to these guidelines is attached as accompanying material for both Chapter 3 and Chapter 4.

2.5 Statistical analysis

2.5.1 GraphPad Prism 9

In addition to storing data in Microsoft Excel (Microsoft Corporation, 2018), using formulas to calculate relative expression levels as well as combine and average data, data was used in GraphPad Prism 9 (GraphPad Software, 2020) for normality, XY and column analysis statistical tests. Alpha significance value was set to $\alpha=0.05$, therefore $P<0.05$ were considered significantly different.

2.5.1.1 Normality tests

Data sets were tested using a number of normality tests provided through GraphPad Prism 9. This was to test if data met the assumptions required for parametric testing, specifically that of normal distribution of data (Sheskin, 2014). Though many columns of data were not significantly different from a normal distribution of data, certain groups were, and so often non-parametric testing was used. Shapiro-Wilk (Thode, 2002), Kolmogorov-Smirnov (Lilliefors, 1967) and D'Agostino and Pearson omnibus K2 (D'Agostino, Belanger and

D'Agostino, Jr, 1990) tests were all used to check the normal distribution of data. Not all data sets passed at least one normality test however, including groups within a multiple comparison passing the same test, and so both parametric and non-parametric testing was used.

2.5.1.2 XY analysis

Simple linear regression (Matthews, 2005) was performed on average dark phase activity for both male and female adult *P. xylostella* and % of activity occurring during dark phase with interpolated y values. Constants for both total activity and activity rate were also overlaid onto these graphs for statistical comparisons between graph curve equations, including testing for differences between male and female slopes. Simple linear regression was also carried out on the last 6h of (s) light phase feeding on both *B. rapa* and *A. thaliana* plants in all conditions, referred to as anticipation slope, with significant slopes compared. Line graphs were also commonly used to show changes in variables over time with SEM error bars used to show variation around average data points.

2.5.1.3 Column analysis

Box and whisker plots were produced with Tukey range for graphical presentation of the spread of data with data points outside the range of the 75^{th} percentile + 1.5x the Interquartile range (IQR) being excluded as a separated point (Tukey, 1977). This was carried out for a number of data columns as it clearly shows the spread of data.

After normality testing, certain non-parametric statistical tests were used for sets of data that were found to be significantly different from a normal distribution. The differences between male and female *P. xylostella* survival in varying locomotor assay conditions, April and June conditions and population cage experiments were tested using Mann-Whitney U tests (Mann and Whitney, 1947). These tests were also used for comparing

differences in total leaf area loss between IP and OP LL3 conditions. Wilcoxon matched pairs signed rank test (MacFarland and Yates, 2016) was used for paired adult data when testing for statistical significances between light and dark phase activity counts of individual moths. Kruskal-Wallis tests with Dunn's multiple comparison post hoc tests were used to compare significant differences of related data sets (Dunn, 1964). This includes the comparisons between locomotor activity under varying light cycles, Larval qPCR relative expression 24h and 48h rhythmic profiles for sulfatase genes and *tim* for 24h. *tim, Sulf3* and *Sulf2+3* required Kruskal-Wallis tests also for comparison of LD days and *Sulf3* for LL day also. OP 24h rhythmic profiles, apart from *Sulf2*, also required Kruskal-Wallis testing along with L/D *tim* and *Sulf3* tests including for L/L rhythms with the latter. Comparisons of *B. rapa* pixel count leaf loss and midday-midnight %, along with 48h weight gain rhythms, were carried out using Kruskal-Wallis tests also. Comparison of midday-midnight leaf area loss from *P. xylostella* larval feeding rhythms on mutant *A. thaliana* was also carried out using such.

Parametric testing was used for all other data sets, meeting the assumptions of such with aforementioned normality tests showing no significant differences from a normal distribution. t-tests (paired t-test for comparisons of same sets of data between different time points) were used to show significant differences between two data columns (Kim, 2015), such as between time points of *per* and *tim*. For comparisons across multiple data sets, including % of total adult activity occurring during dark phase which was found to be normally distributed compared to other adult *P. xylostella* locomotor activity data sets, a one-way ANOVA with Tukey's multiple comparisons analysis was used (McHugh, 2011). In addition, a two-way ANOVA was used to calculate proportional effect of variables on the relative expression of *P. xylostella* genes.

2.5.2 Estimation statistics

Estimation statistics seek to demonstrate the relative effect sizes and uncertainty of such in a clearer way than only through alpha significance testing (Ho et al., 2019). The software provided by the estimation statistics paper was used to generate Tufte slopegraphs depicting the differences between activity in certain conditions, the two-sided permutation t-test produced with these graphs was used to show significant difference between these data sets of both male and female adult *P. xylostella*.

2.5.3 CircaCompare

CircaCompare analysis (Parsons et al., 2020) was used in R (R Core Team, 2021), circacompare R package title. CircaCompare analysis allows for the calculation and comparisons of rhythmic profiles using certain rhythmic features (mesor, Amplitude and Phase) to compare rhythmic patterns between groups of data through cosinusoidal curve fitting. 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 time points where the peak of the fitted cosinusoidal curve occurs. Differences between adult male and female data was compared using CircaCompare along with differences between April and June mimicking semi-field conditions. CircaCompare was also used to compare larval feeding and weight gain rhythms between light and entrainment conditions and between feeding on mutant and WT *A. thaliana*. Relative expression rhythms of clock and glucosinolate sulfatase genes were also investigated using CircaCompare, showing differences between L/D, D/D and L/L expression rhythms.

Chapter 3 Daily Behavioural and Molecular Rhythms in Adults of a Long-term Lab Culture of the Lepidopteran Agricultural Pest the Diamondback Moth (*Plutella xylostella*)

3.1 Abstract

The Diamondback Moth, *Plutella xylostella,* is arguably the most economically impactful and widespread lepidopteran pest, having achieved a cosmopolitan distribution and causing ~$5 billion worth of damage annually. 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 daily rhythms in both adult behaviour and clock gene expression. Under simulated natural cycles of light and temperature the locomotor behaviour of individual moths was predominantly nocturnal and indicated a temperature preference. Individual adult activity across different photoperiods at constant temperature revealed both light-repressed and homeostatic regulation, but no anticipatory activity associated with circadian regulation. Moreover, no behavioural rhythmicity was detected under constant conditions in either individual or group assays, further confirming a lack of circadian control of adult locomotor behaviour. In contrast, real-time quantitative PCR analyses of the same long-term lab culture of *P. xylostella* demonstrated both diel and circadian rhythms for transcripts of the clock genes *period* and *timeless* indicating the presence of a daily timekeeping mechanism. 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.

3.2 Introduction

Food security and the efficient use of agriculture are critical issues that need to be addressed as climate change and an exponentially growing human population put pressure on arable land, threatening global famines before the end of the century (Ericksen, Ingram and Liverman, 2009, Cribb, 2010). A key factor in food production is the control of pest species, with the effects of pests underestimated (Bradshaw et al., 2016) and increasing in severity as the climate changes (Early et al., 2016, Paini et al., 2016, Deutsh et al., 2018, Savary et al., 2019). *P. xylostella* (The Diamondback moth) is a global pest species, having achieved a cosmopolitan distribution with a seasonally expanding range (Talekar and Shelton, 1993, Furlong, Wright and Dosdall, 2012). *P. xylostella* is a specialist brassica (cruciferous vegetables) herbivore (Ratzka et al., 2002) in the lepidopteran order, that causes worldwide losses of ~$5 billion annually (Zalucki et al. 2012, Furlong, Wright and Dosdall, 2012), up from ~$1 billion in the 1990's. This makes *P. xylostella* arguably the world's most costly lepidopteran pest (Talekar and Shelton, 1993). *P. xylostella* poses a challenge to farmers due to rapid proliferation in colonized fields, able to produce up to ~400 eggs per female (Sarfraz et al., 2011) and complete multiple life cycles per season (Crafford and Chown, 1990, Talekar and Shelton, 1993). These life history traits feed into *P. xylostella's* rapid pesticide resistance gain, also facilitated by a number of genetic factors (Tabashnik et al., 1997, Xia et al., 2015), including that of gut microbiota (Xia et al. 2018). These factors have led to control failures with farmers in some regions facing >90% crop losses (Srinivasan and Krishnamoorthy, 1992, Verkerk and Wright, 1996), leading to heavy use of broad-spectrum pesticides, that not only help fuel pesticide resistance gain (Dover and Croft, 1986, NRC, 2000), but damage natural ecosystems and human health (Pimentel and Edwards, 1982, Aydinalp and Porca, 2004, RCEP, 2005, Jepson et al., 2014, Mahmood et al., 2015). IPM (Integrated Pest Management) strategies are being implemented to help diminish the use of pesticides and reduce *P. xylostella's* impact

(Sayyed et al., 2002, Birch, Begg and Squire, 2011, Philips et al., 2014), however, the uptake of these strategies has been limited, with the prohibitive complexity of treatments causing farmers to fall back onto broad-spectrum pesticides (Sarfraz, Keddie and Dosdall, 2007, Norton et al., 2008, Uthamasamy et al., 2011). 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 (Foster and Harris, 1997, Cook, Khan and Pickett. 2007, Breda et al., 2013).

The circadian clock acts as an endogenous time keeping mechanism to control physiological processes in organisms to best fit the daily ~24 hour (h) rhythms of the planet (Pittendrigh, 1993, Foster and Kreitzman, 2005). The '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 but can be entrained by such. The molecular clock, as shown in the fruit fly *Drosophila melanogaster*, uses the proteins PER (Period) and TIM (Timeless) as part of the TTFL model, together forming a heterodimer that can be broken by a light receptive pathway that degrades TIM. The PER:TIM complex inhibits its own expression generating the core negative feedback loop. These cycles are maintained in the absence of inputs through interactions with other TTFL involving the proteins CLK (Clock) and CYC (Cycle), which is entrained via light sensitive pathways (Zeng et al., 1996, Huang, 2018). The *D. plexippus* circadian clock, which is the best studied lepidopteran model, differs from the model organism for arthropods (*D. melanogaster*), having two CRY (Cryptochrome) proteins active in the PER/TIM TTFL. Mammalian-like (m)CRY acts as a negative regulator, in the complex that represses *per, tim* and in this case m*cry* expression (Zhu et al., 2005, Zhu et al., 2008).

Circadian rhythms have widespread impact on gene expression, with research showing ~40% of coding genes exhibit circadian rhythms in mice (Zhang et al., 2014) and ~35% in plants (Michael and McClung, 2003). 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 (Janszky and Ljung, 2008, Kim et al., 2011, Scheiermann, Kunisaki and Frenette, 2013, Curtis et al., 2014). Lepidopteran circadian mechanisms have been shown to influence numerous behaviours including life stage cycles, oviposition (Sauman and Hashimi, 1999) and seasonal migrations (Froy et al, 2003). Such changes in behaviour may help *P. xylostella* to best suit its local environments and react to changes in such to maintain fitness. A key response being migration, which can be reliant on changes in photoperiod (Robart, McGuire and Watts, 2018, Sockman and Hurlbert, 2020) and temperature (Klinner and Schmaljohann, 2020, Burnside et al., 2021). *P. xylostella* experiences rapid changes in such factors due to distances migrated, travelling near 1000km in less than 48hrs (Coulson et al., 2002). The way in which *P. xylostella* controls and modulates its behaviour when invading these new regions and after invasions have taken place may be important for informing new control measures as well as complimenting and improving the efficacy of previously implemented IPM strategies (Ferguson et al., 2015). It has been shown that *P. xylostella* behaviours such as mating, oviposition and adult emergence show significant diel rhythms (Pivnick et al., 1990), however other research has shown a lack of responses to photoperiods (Campos, 2008).

Therefore, an important aspect of *P. xylostella* behaviour will be its rhythmicity. To address this as well as provide evidence for underlying control mechanisms, lab-reared *P. xylostella* locomotor activity was studied under a range of environmental conditions. In addition, the molecular context of observed diel rhythms was explored by determining transcript profiles of the *P. xylostella* clock genes *per* and *tim*.

3.3 Results

3.3.1 Male-female differences and survivorship

The survivorship of *P. xylostella* male and females in light cycle and environmental mimic conditions is shown in Table 3.1, where there is some variation of survival between light cycle conditions. Only males were used in environmental mimic conditions.

Table 3.1 Adult *P. xylostella* survival across conditions.

Adult *P. xylostella* show variation in survival between light cycle conditions but a significant difference between average male and female survival in light cycle locomotor assays. An equal number of male and female *P. xylostella* were originally placed in light cycle TriKinetics assays. Table shows not all moths produced data to complete a 6 day average. Setup conditions were in constant dark (D/D) and light (L/L) with cycles of differing hours (h) of light and dark (L/D) denoted as 12/12 for 12 h of light and 12 h of dark. April and June UK environmental mimicking conditions were also used.

Condition	% that survived 6 days (Males)	% that survived 6 days (Females)	Final n Male / Female
D/D	76.7	60.7	23 / 17
12/12	55.0	24.5	33 / 13
6/6/6/6	68.6	29.4	35 / 10
14/10	46.5	25.6	20 / 10
16/8	87.1	40.0	27 / 12
18/6	78.6	27.5	33 / 10
20/4	93.3	61.3	28 / 19
L/L	83.9	37.0	26 / 10
April	54.1	n/a	20 / n/a
June	66.7	n/a	20 / n/a

Overall, females had significantly lower survival on average across light cycle conditions after 6 days of data collection. The number of male and female *P. xylostella* in the repeats for each is therefore variable. Analysis of differences in locomotor activity between male and female *P. xylostella* is shown in Fig. 3.1.

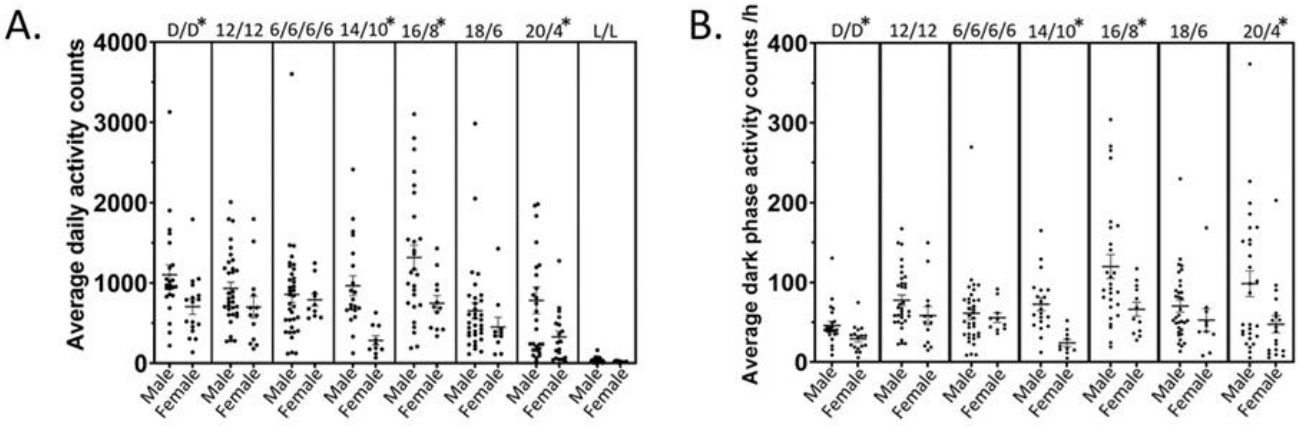


Figure 3.1 Locomotor activity counts compared between adult *P. xylostella* sexes.

Female *P. xylostella* produce significantly less activity counts than males in multiple light cycle conditions. A & B show nested individual value scatter plots for each light cycle condition between male and females with horizontal bar showing mean with SEM error bars. A) shows total average activity over 24h of adult *P. xylostella*, B) shows total dark phase average activity divided by dark phase period in h. * shows when male and female activity is significantly different

Average total daily activity counts were significantly lower from female *P. xylostella* in D/D (constant dark) conditions, with 14/10, 16/8 and 20/4 reduced dark phase light cycle conditions also showing significantly lower activity counts compared to males. The same significant differences were found for rate of dark phase activity in these specific conditions, confirming that often female *P. xylostella* may have lower activity counts and rate of activity in addition to survivorship in conditions.

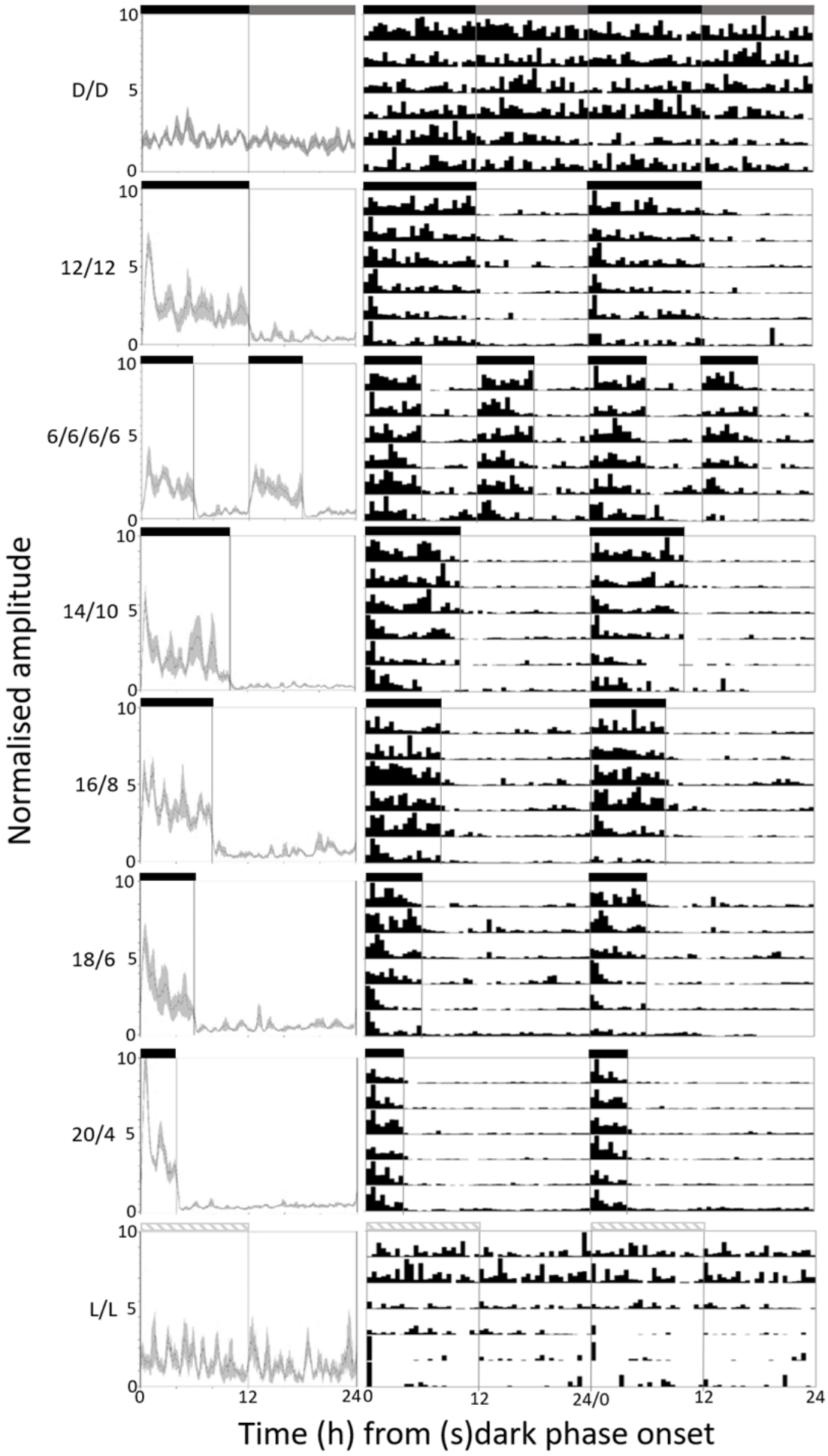

Normalised amplitude
D/D
12/12
6/6/6/6
14/10
16/8
18/6
20/4
L/L
10
5
0
0
12
24
0
12
24/0
12
24
Time (h) from (s)dark phase onset

Figure 3.2 Adult female *P. xylostella* locomotor activity rhythms.

Adult *P. xylostella* exhibit consistent rhythmic locomotor activity patterns in varying light cycle conditions and arrhythmia in constant conditions. Average activity profiles (Left) show average activity over 24hrs of the 6 day average activity of a number of *P. xylostella* adult females, shown in Table 3.2. Time is plotted along x-axis in hours (h), starting at dark phase and subjective (s) dark phase onset with normalized amplitude along y-axis. Central line shows average activity with the shaded area showing SEM. The black bar along the top of individual graphs shows dark phase with shaded bars showing s light phase for constant dark and cross hatched bars s dark phase for constant light. Double plotted actograms (Right) show average activity of moths over 6 days normalized against the next day's activity in 30-minute bins of counts. 1st then 2nd day shown across first row of actograms, then 2nd to 3rd and so on normalized against each other within a row.

Female locomotor activity demonstrates dark phase centred activity with over 80% of activity occurring during dark phase in 12/12, 6/6/6/6 and 14/10 conditions shown in Fig. 3.2 and 3.3C. Activity during constant conditions subjective (s) dark phase is more even spread averaging closer to 50% being significantly lower than the aforementioned light cycle conditions. There is a relatively pronounced dark phase onset peak in light cycle conditions with Fig. 3.3D showing how as the total dark phase period decreases the proportion of total activity concentrated into the h's immediately after dark phase onset increases. However, Fig. 3.3B shows how average dark phase activity rate doesn't increase as much as proportion in this initial dark phase time period in the short dark phase conditions such as 18/6 and 20/4.

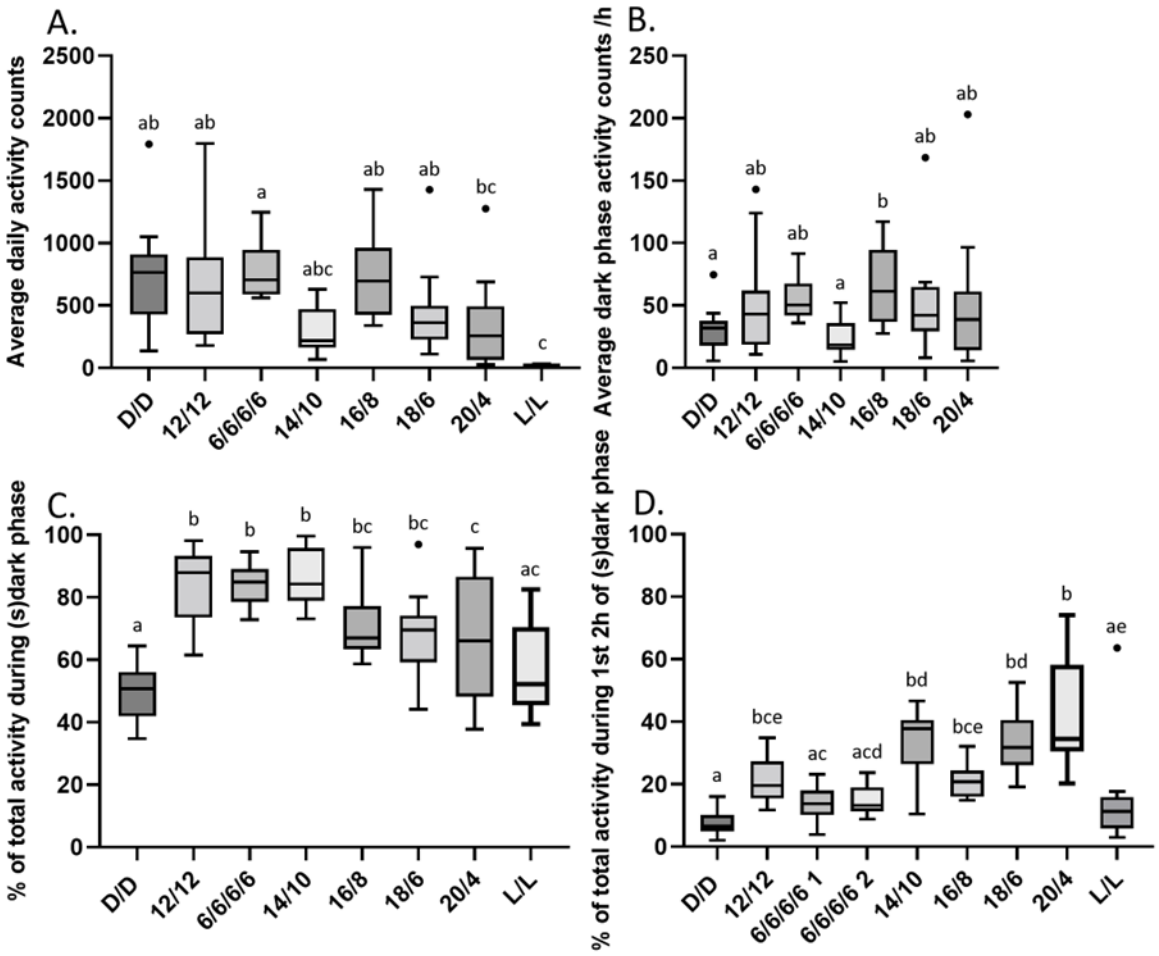


Figure 3.3 Locomotor behavioural analysis of adult female *P. xylostella* under different lighting conditions.

Adult female *P. xylostella* display evidence of homeostatic control of activity through the maintenance of an average amount of total activity across light cycle conditions by modulating activity. The box and whisker plots demonstrate shifts in features of locomotor activity over conditions, showing A) total average activity over 24 h of adult P. xylostella in varying light cycle conditions, B) total dark phase average activity divided by dark phase period in h, C) average % of total activity occurring during (s) dark phase, D) average % of total activity occurring during first 2h 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.

Other than in L/L (constant light) conditions, all other light cycle conditions had no significance difference in average daily activity to each other shown in Fig. 3.3A. Even

while Fig. 3.3C shows ~80% of activity occurred during the dark phase in 12/12 conditions. When this is split into 12h periods of 6/6 (6h of light and 6h of dark) the average daily activity is also significantly higher than 20/4. Periodogram results shown in Table 3.2 relatively uniform % of female moths that were found to be rhythmic (minus 18/6), average period lengths and relative rhythmic power (RRP).

Table 3.2 Female *P. xylostella* locomotor activity periodogram data.

Data from Fig. 3.2, female *P. xylostella* under varying light cycle conditions with ±SEM. % of moths that had significant periodogram rhythms with average period length and RRP of such. (n) number of repeats, (h) hours. # shows female L/L only has 1 rhythmic moth. RRP calculated using ratio between periodogram dominant period likelihood amplitude and significance threshold for h length of specified period.

Female Condition (n)	% rhythmic	Period length (h)	RRP
D/D (17)	17.6	25.25 ±0.7	1.05 ±.01
12/12 (13)	76.9	24.15 ±0.4	1.41 ±.16
6/6/6/6 (10)	80.0	23.9 ±0.2	1.40 ±.07
14/10 (10)	90.0	23.8 ±0.1	1.38 ±.06
16/8 (12)	91.7	25.1 ±0.9	1.16 ±.04
18/6 (10)	40.0	23.6 ±0.1	1.24 ±.08
20/4 (19)	89.5	24 ±0.0	1.59 ±.11
L/L (10)	10.0	24.0#	1.40#

Both male and female *P. xylostella* in constant light cycle conditions produced arrhythmic data with only light cycle conditions producing rhythmic data for CircaCompare analysis shown in Table 3.3. Though measures of behavioural rhythmicity in other conditions are similar and not significantly different between sexes, excluding 6/6/6/6 mesor and

amplitude values, female *P. xylostella* are overall less active, often producing significantly lower average activity rates which would affect total averages. Due to this, male and female data was separated for locomotor activity analysis, focusing on males for Fig. 3.4-3.9 and Table 3.4-3.6, due to higher survivorship in set ups and activity rates, potentially making it easier to identify changes and fit with qPCR data that had to be collected using only males.

Table 3.3 Male vs female adult *P. xylostella* activity rhythm comparison.

Adult *P. xylostella* male and females show similar rhythms with few significantly different rhythmic features (6/6/6/6 Mesor and Amplitude) denoted by *. 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.

Condition	M Rhythm *P*	F Rhythm *P*	M Mesor	F Mesor	M Amplitude	F Amplitude	M Phase peak	F Phase peak
12/12	4.05^{-8}	6.61^{-8}	1.67	1.44	1.48	1.24	4.16	5.00
6/6/6/6	3.44^{-13}	9.37^{-15}	1.51	1.20*	1.49	1.11*	5.47	6.23
14/10	6.01^{-6}	3.38^{-8}	1.09	1.05	1.08	1.21	2.73	4.23
16/8	1.31^{-8}	3.58^{-11}	1.50	1.59	1.76	1.68	3.17	3.39
18/6	2.50^{-6}	1.86^{-6}	1.06	1.11	1.15	1.08	2.29	2.06
20/4	1.50^{-4}	2.72^{-5}	1.11	1.05	1.27	1.29	1.52	1.49

3.3.2 April and June environmental mimic assay

In Fig. 3.4A a distinct peak in activity occurs at complete dark phase onset with a minor peak occurring roughly 20h after dark phase onset, corresponding to both high temperatures and beginning of light transition phase in April conditions. Overall, light

transition phases and temperature have a more minimal effect on activity profile than complete lights off and presence of any light.

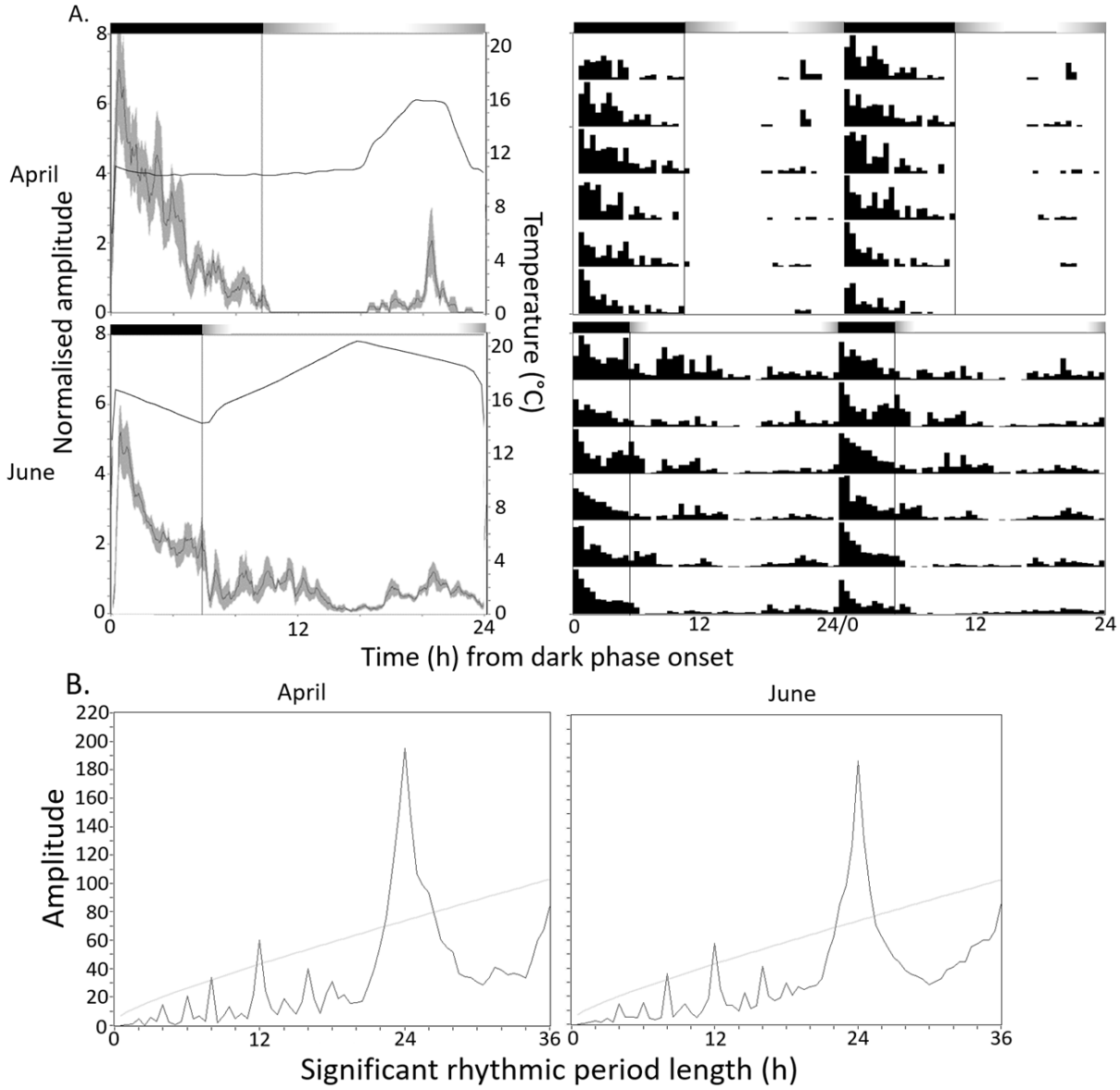


Figure 3.4 Male adult *P. xylostella* locomotor activity under UK April and June semi-field conditions.

Male adult *P. xylostella* locomotor activity under simulated UK April and June environmental conditions show rhythmic activity peaking at dark phase onset.

A. (Left) Average activity profiles show activity over 24h 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 plotted on graph to show temperature cycle with separate y-axis to the right. (Right) Double plotted 24h actograms show average activity of moths over 6 days normalized against the next day's activity in 30-minute bins of counts. Recorded environmental profile shown in Fig. 2.2.

B. Average periodograms showing significant rhythm period lengths found from the data in April (left) and June (right) mimic conditions with period length along x-axis and amplitude of significant rhythm period on y-axis. Pale grey line shows significance threshold for period lengths and dark line shows amplitude of rhythmic period likelihood from data.

After lights on in June conditions an increase in activity occurs for a period of time as the temperature increases before the activity reaches its trough around peak temperature, shown in Fig. 3.4. These more minor changes in light phase activity level follow closely with periods of changing temperature. Total activity, initial peak activity, dark phase activity rate and rhythmic features are not significantly different between conditions as shown in Fig. 3.5 and Table 3.4, however total activity that occurs during the dark phase is significantly different with ~80% of total activity occurring during the 10h of complete dark phase in April conditions, similar to results shown in Fig. 3.3 and 3.7.

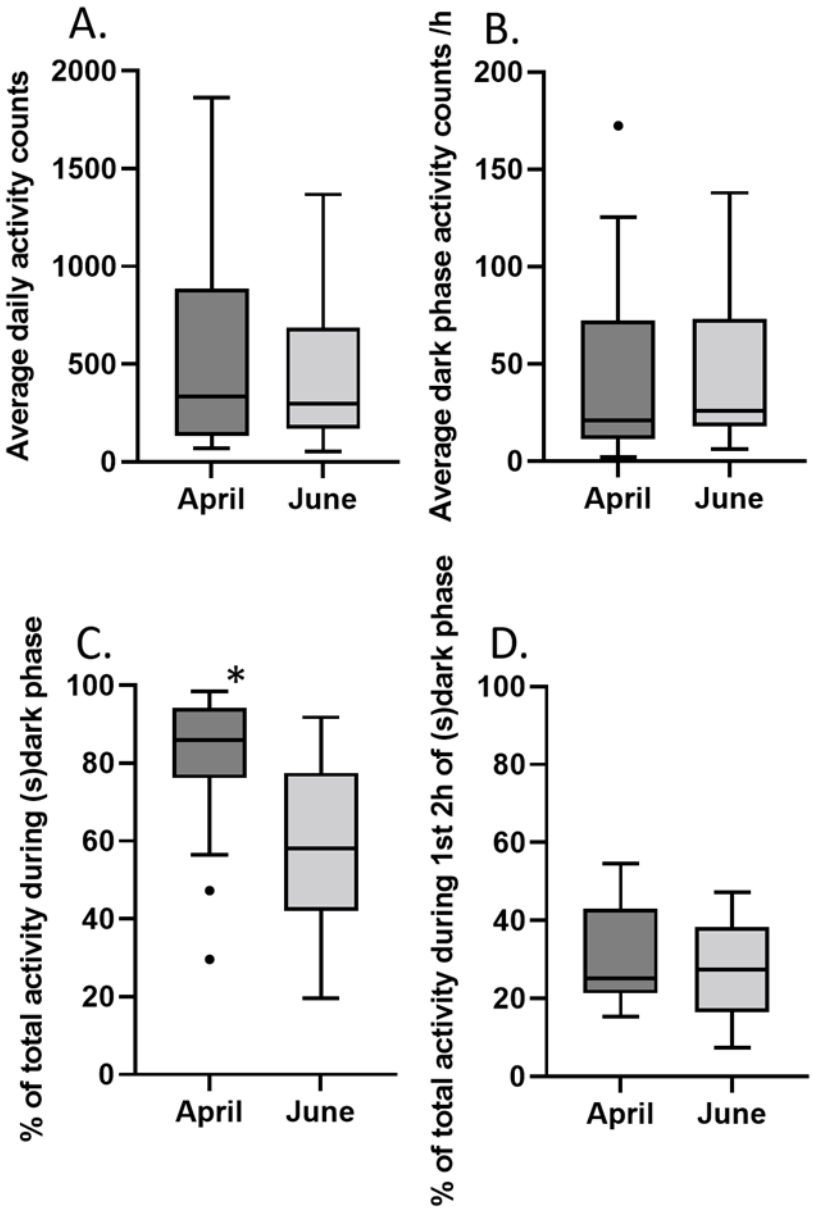


Figure 3.5 Locomotor behavioural analysis of adult *P. xylostella* under April and June semi-field conditions.

Adult! *P. xylostella* show similar The box and whisker plots demonstrate shifts in features of locomotor activity over conditions, showing A) total average activity over 24 h of adult P. xylostella in varying light cycle conditions, B) total dark phase average activity divided by dark phase period in h, C) average % of total activity occurring during (s) dark phase, D) average % of total activity occurring during first 2h 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 3.4 April and June semi-field conditions rhythmic data.

(n) repeat number, (h) hours. % 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 April and June condition. 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.

Condition (n)	% Rhythmic	Period length (h)	Rhythm *P*	Mesor	Amplitude*	Phase peak	RRP
April (20)	90%	24.1 ±0.1	$1.10x10^{-8}$	1.07	1.59	2.77	1.68 ±.09
June (20)	80%	24.1 ±0.1	$1.08x10^{-7}$	1.14	1.01	2.52	1.56 ±.13

June mimicking conditions where there is only 5.5h of dark phase shows only ~58% of total activity is being expressed during the dark phase. This level of dark phase focused activity is lower than that seen in 20/4 conditions shown in Fig. 3.3 and 3.7, towards D/D and L/L conditions arrhythmic results, however, as can be seen in Fig 3.4 and Table 3.4, June conditions produce clear rhythmic results. When April and June activity rhythms are compared, though both are similar, April conditions have a significantly higher amplitude.

3.3.3 Varying light cycle conditions assay

The deeper comparisons between light cycle conditions further explores adult *P. xylostella* activity and helps demonstrates the effects of light on activity and how *P. xylostella* adults may modulate their behaviour over time.

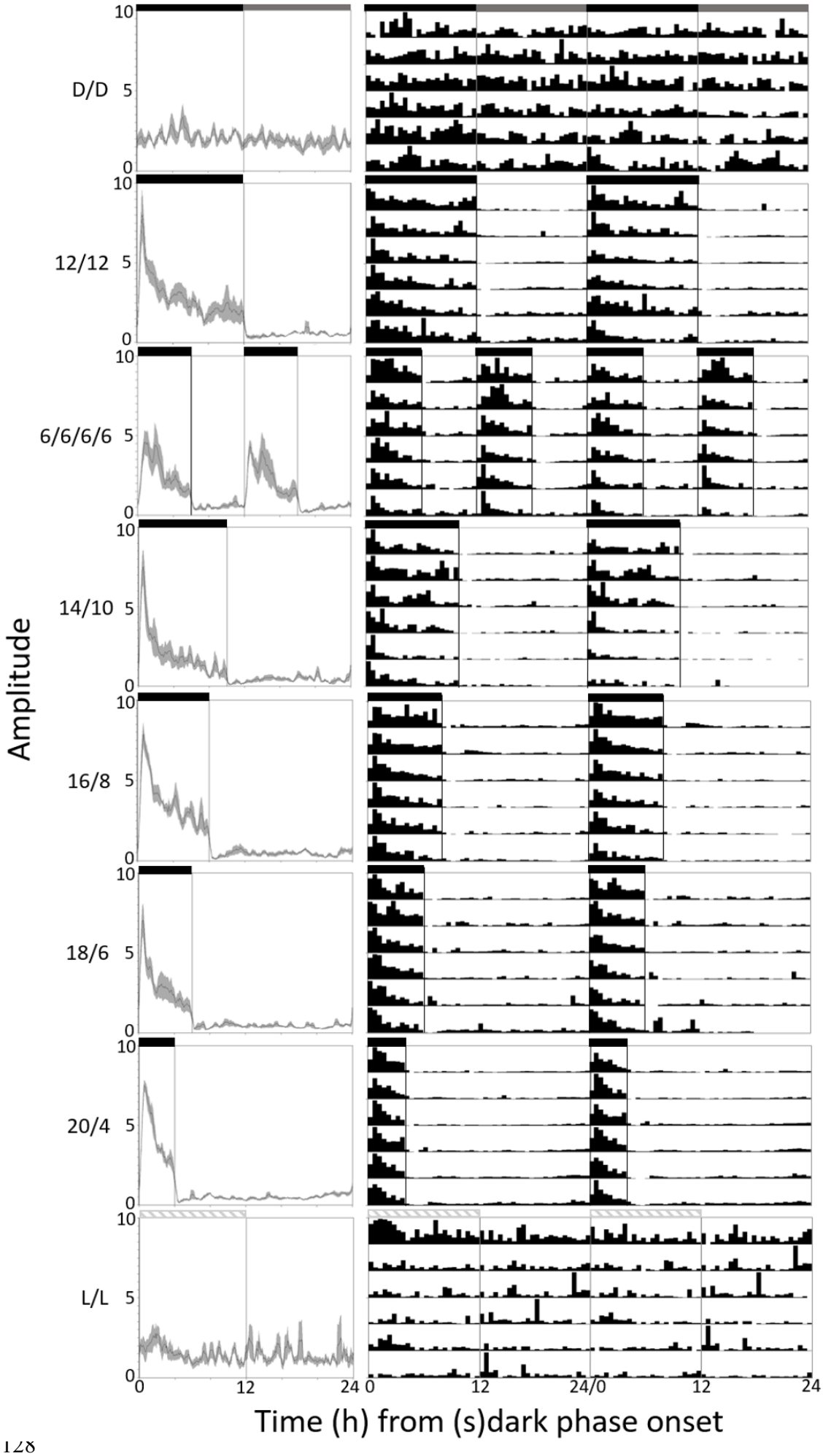

Amplitude
D/D
12/12
6/6/6/6
14/10
16/8
18/6
20/4
L/L
10
5
0
0
12
24
24/0
Time (h) from (s)dark phase onset

Figure 3.6 Locomotor behavioural analysis of adult male *P. xylostella* under different lighting conditions.

Adult *P. xylostella* exhibit consistent rhythmic locomotor activity patterns in varying light cycle conditions and arrhythmia in constant conditions. Average activity profiles (Left) show average activity over 24hrs of the 6 day average activity of a number of *P. xylostella* adult males. Time is plotted along x-axis in hours (h), starting at dark phase and subjective (s) dark phase onset with normalized amplitude along y-axis. Central line shows average activity with the shaded area showing SEM. The black bar along the top of individual graphs shows dark phase with shaded bars showing s light phase for D/D and cross hatched bars s dark phase for L/L. Double plotted actograms (Right) show average activity of moths over 6 days normalized against the next day's activity in 30-minute bins of counts.

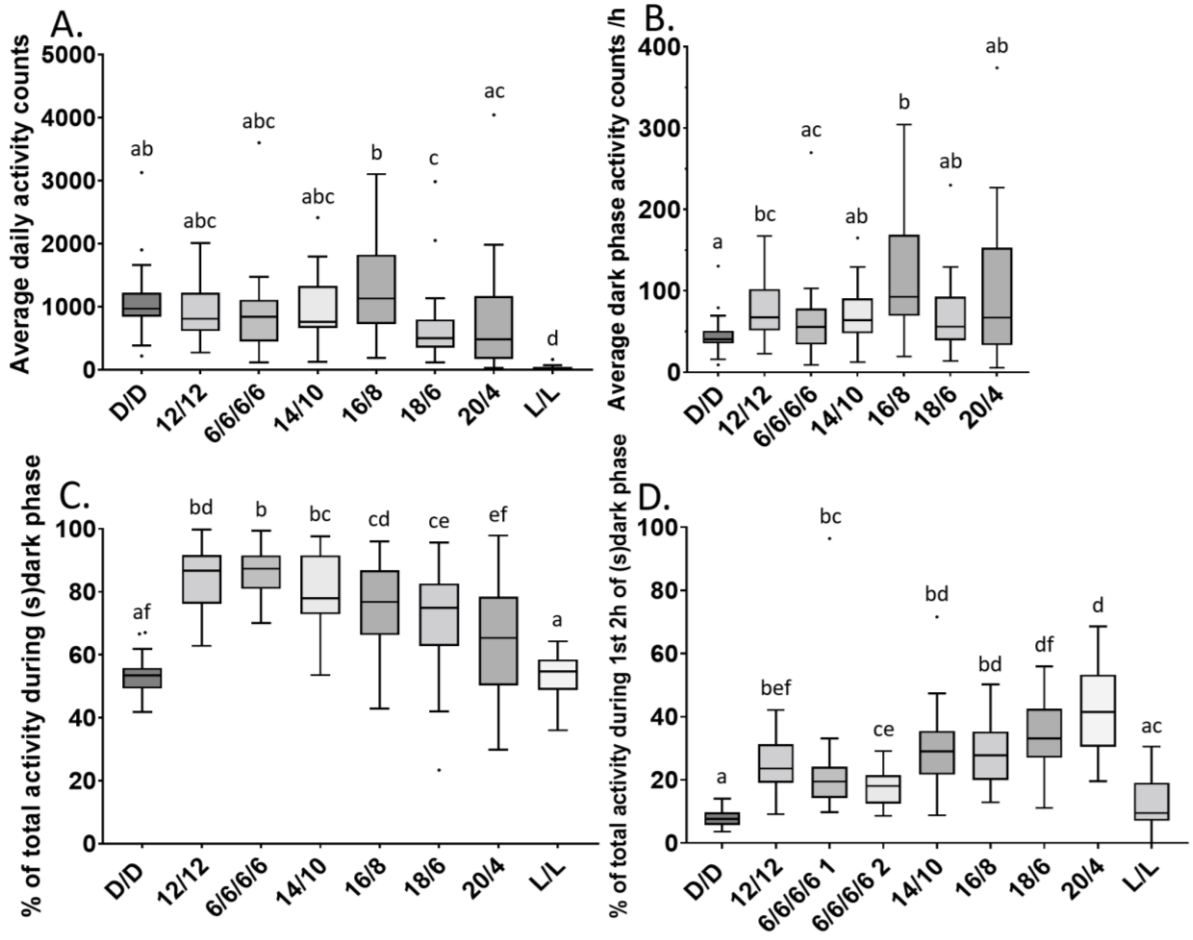


Figure 3.7 Locomotor behavioural analysis of adult *P. xylostella* under different lighting conditions.

Adult *P. xylostella* display maintenance of an average amount of total activity across light cycle conditions by modulating activity. The box and whisker plots demonstrate shifts in features of locomotor activity over conditions, showing A. total average

activity over 24 h of adult *P. xylostella* in varying light cycle conditions, B. total dark phase average activity divided by dark phase period in h, C. average % of total activity occurring during (s) dark phase, D. average % of total activity occurring during first 2h 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.

Results shown in Fig. 3.6 demonstrate dark phase centred activity in adult *P. xylostella*, with the majority of activity occurring during the dark phases of varying lengths across a 24h light cycle period, while constant conditions show arrhythmic activity profiles. The concentration of activity into the dark phase is shown in Fig. 3.7C, showing this ranges between ~85% of activity at 12h of dark phase, significantly decreasing to ~64% of activity with 4h of dark phase in light cycle conditions. Table 3.5 shows how in light cycle conditions the majority of male *P. xylostella* were rhythmic, maintaining this dark phase focused activity diel cycles with ~24h period length. In contrast, constant conditions have activity that is more evenly spread, lacking a clear concentration of activity into any single 12h phase with ~53% of activity in circadian dark phases, significantly lower than conditions with light cycles with very few moths calculated as maintaining rhythmic diel activity. 6/6/6/6 conditions also show no clear preference for any single 12h phase.

Typically in light cycle conditions, after a peak in activity counts from dark phase onset, activity decreases to a medium level of activity during the dark phase before cutting off at light phase onset, usually forming the trough of activity. Periodogram results showed only 1 moth in D/D could be counted as rhythmic while 3 was found for L/L therefore stats could be carried out on L/L rhythmicity in Table 3.5. This may have occurred due to the low quantity of activity counts in the L/L data set. The proportion of rhythmic moths for light cycle conditions also ranged from 75% rhythmic for 14/10 up to 96.4% rhythmic for 20/4. 20/4 also had a significantly higher RRP compared to all other conditions. Excluding

D/D, all conditions had non-significantly different significant rhythmic period lengths. When light cycle and constant conditions were averaged and processed through CircaCompare only D/D and L/L were calculated as arrhythmic, data shown in Table 3.3.

Table 3.5 Male *P. xylostella* locomotor activity periodogram data.

Periodogram data from Fig. 3.6. Male *P. xylostella* under varying light cycle conditions with ±SEM. (n) number of repeats, (h) hours. # indicates that male D/D and L/L only had 1 rhythmic moth and * indicates a significantly higher RRP for 20/4 compared to other conditions. RRP calculated by using the ratio between amplitude and significance threshold.

Male Condition (n)	% rhythmic	Period length (h)	RRP
D/D (23)	4.3	32.5#	1.01#
12/12 (33)	84.8	23.9 ±0.1	1.38 ±.05
6/6/6/6 (35)	85.7	24.1 ±0.3	1.47 ±.10
14/10 (20)	75.0	23.9 ±0.1	1.33 ±.08
16/8 (27)	85.2	24.0 ±0.1	1.51 ±.08
18/6 (33)	87.9	23.8 ±0.2	1.47 ±.08
20/4 (28)	96.4	24.0 ±0.1	1.75 ±.07*
L/L (26)	11.5	27.2 ±2.1	1.07 ±.03

Results, shown in Fig. 3.8, demonstrate how group populations of mixed-sex adult *P. xylostella* exhibit similar behavioural rhythms in both 12/12 and D/D conditions to those shown in Fig. 3.6.

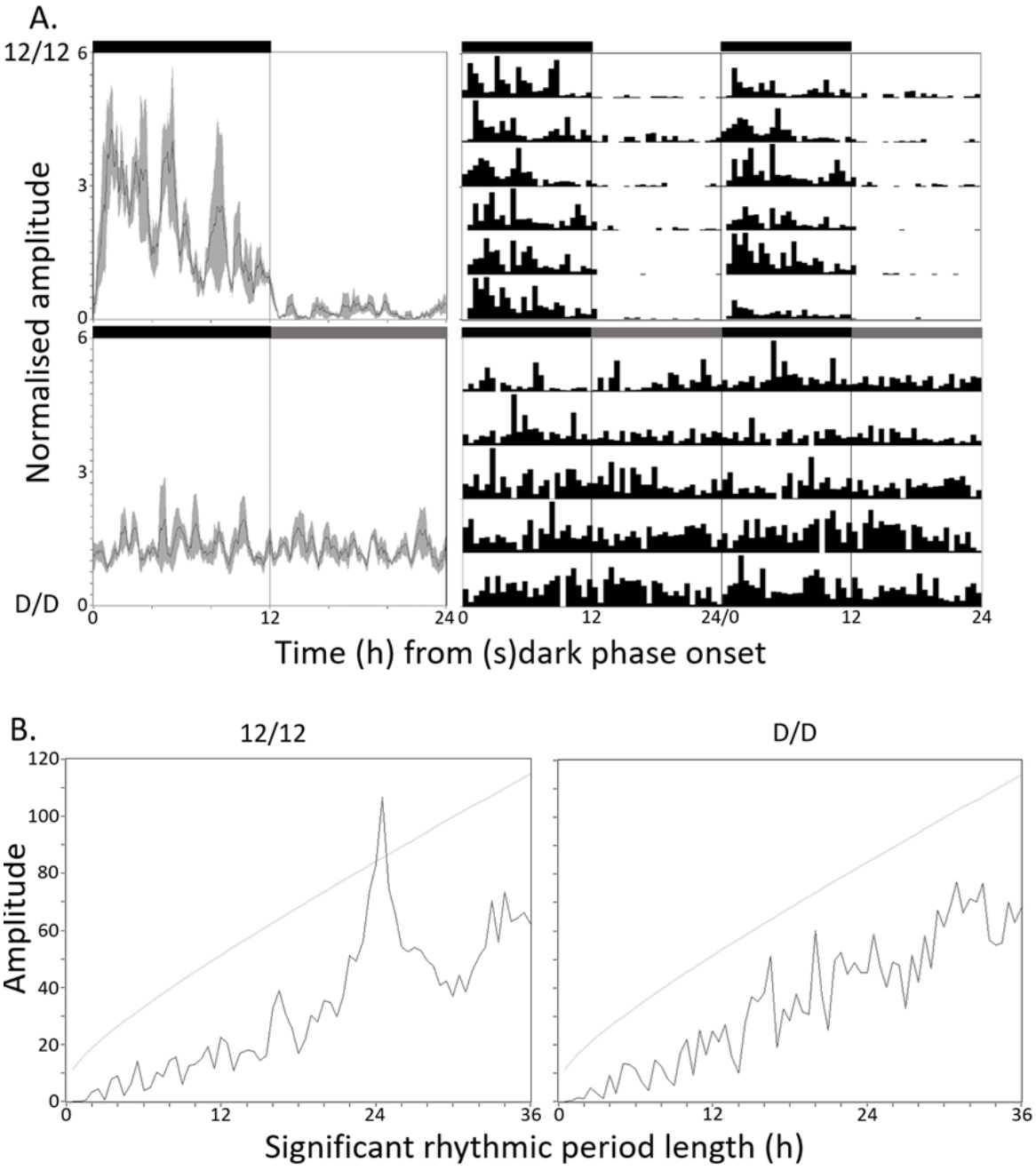


Figure 3.8 Adult *P. xylostella* population assay rhythms.

Mixed-sex groups of *P. xylostella* show rhythmic activity proximal to food source in 12/12 light cycle conditions and arrhythmia in constant dark.

A. Average activity profiles (Left) show the average activity over 24hrs of the 6 day average activity of a group of 30 male and female *P. xylostella* adults. Time is plotted along x-axis in hours (h), starting at dark phase and subjective (s) dark phase onset with normalized amplitude along y-axis. Central line shows the average activity with the shaded area showing SEM. The black bar along the top of individual graphs shows dark phase with shaded bars showing s light phase for

D/D. Double plotted actograms (Right) show average activity of moths over 6 days normalized against the next day's activity in 30-minute bins of counts.

B. Average periodograms showing significant rhythm period lengths found from the data in 12/12 (left) and D/D (right) conditions with period length along x-axis and amplitude of significant rhythm period on along y-axis. Pale grey line shows significance threshold for period lengths and dark grey ling showing amplitude of rhythmic period likelihood from data.

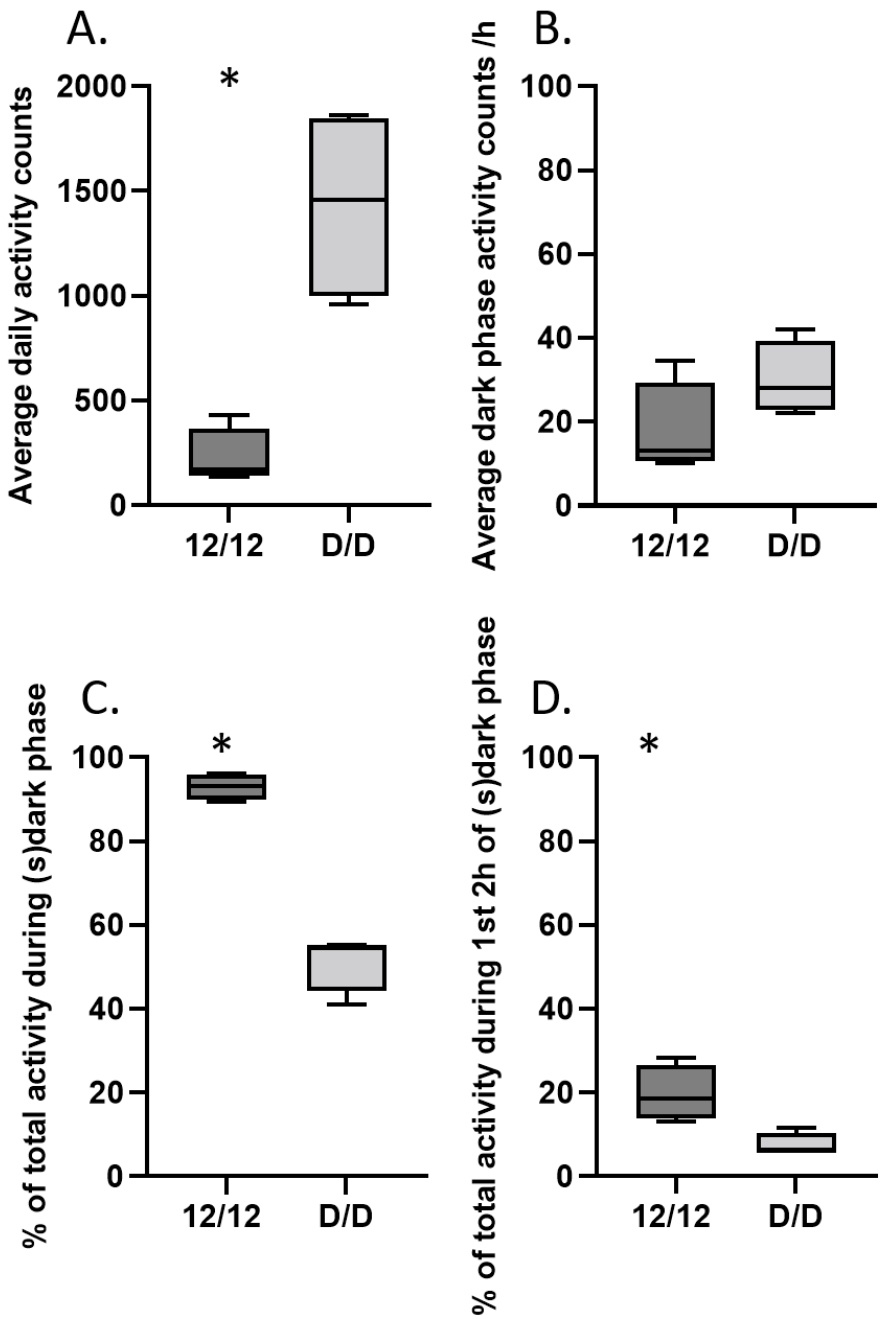


Figure 3.9 Adult population cage behavioural analysis between 12/12 and D/D conditions.

Adult *P. xylostella* display significant differences between the average amounts of activity between 12/12 and D/D conditions. The box and whisker plots demonstrate shifts in features of locomotor activity over conditions, showing A. total average activity over 24 h of adult *P. xylostella* in varying light cycle conditions, B. total dark phase average activity divided by dark phase period in h, C. average % of total activity occurring during (s) dark phase, D. average % of total activity occurring during first 2h of (s) dark phase. Data points outside Tukey range (1.5XIQR (Interquartile range)) are shown. Mann-Whitney U test results show significant differences from each respective data set through *.

The recorded activity proximal to food source, where moths were free to move and interact with each other, showed ~93% of counts taking place during dark phase in 12/12 and only ~51% in subjective (s) dark phase in D/D. Total activity in D/D was also shown to be significantly higher than in 12/12, shown in Fig. 3.9A, in contrast to the findings from Fig. 3.7.

Table 3.6 Adult population assay periodogram data.

Periodogram data from Fig. 3.8 ±SEM. (n) number of repeats, (h) hours.

Condition (n)	% rhythmic	Period length (h)	RRP
12/12 (4)	100	24.1 ±0.2	1.44 ±.21
D/D (4)	0	n/a	n/a

The 20/4 condition has the largest range in % of activity occurring during the dark phase and is the only light cycle condition with a range that extends below 50% of activity. Though the majority of moth activity is still expressed during dark phase, with the range nearing up to 98% of activity, there was a number of moths that could no longer maintain such with only 4h of dark phase. This is also evident from 20/4 not being significantly different from D/D.

Fig. 3.7A shows *P. xylostella* exhibit <5% the activity of D/D in L/L, having significantly lower total activity than all other conditions. However, as dark phase decreases in light cycle conditions from 12h to 4h, the total average activity doesn't significantly decrease, with 18/6 conditions only significantly lower than 16/8 and D/D, with 20/4 only less than 16/8. More notably, when 24h of dark phase is provided in D/D, though over 80% of activity is dark phase centred in 12h dark phase conditions, there is no significant increase in total activity.

Changes in the peak concentration of activity, shown within the first 2h of dark phase onset in Fig. 3.6 light cycle conditions, varies to a greater extent than total or dark phase activity/h. Shown in Fig. 3.7D, the sharp peak seen in the activity profiles at the onset of dark phase increases as a proportion of total activity from light cycle conditions. This increases from conditions with higher h of dark phase to those with less, with 18/6 and 20/4 having a significantly higher proportion of their activity occurring in the 2h from dark phase onset than both 6/6/6/6 peaks and 20/4 more than 12/12, compared to a more uniform rate of activity during dark phase shown in Fig. 3.7B. 6/6/6/6 shows no significant difference between their first 2h proportional peaks from L/L conditions in Fig. 3.7D, though L/L's activity counts are significantly lower. D/D activity 2h from s dark phase onset as a proportion of total activity is significantly less than all light cycle conditions.

The rate of activity during the dark phase, shown in Fig. 3.7B, increases from D/D to 16/8 conditions, despite this only D/D and 6/6/6/6 is significantly lower than the 16/8 rate. After 16/8, the average drops down from ~120 counts/h to ~70 counts/h in 18/6 before increasing again to ~98 counts/h in 20/4. Both 18/6 and 20/4 are not significantly different from any other condition however.

Fig. 3.10 shows the difference between recorded average dark phase activity and trend lines of *P. xylostella* adults, assuming both a constant dark phase activity rate as dark phase length changes and a constant total amount of dark phase activity.

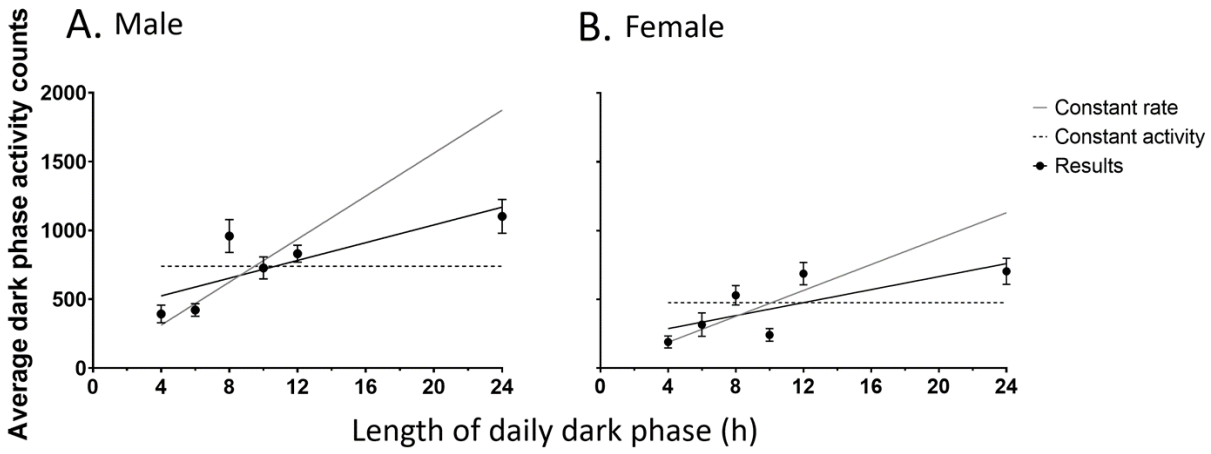


Figure 3.10 Adult male and female *P. xylostella* average dark phase activity counts linear regression.

Both male and female *P. xylostella* adults show an intermediary trend line (solid black) between a constant rate of activity per h during dark phase (solid grey) and constant total dark phase activity (dotted line). Scatter plot with simple linear regression lines showing average dark phase activity counts results against h of dark phase in 24h light cycles and D/D for A. Male and B. Female, *P. xylostella* adults. Black dots show mean results values with SEM error bars.

A significant linear trend of $R^2 = .64$, $Y = 33.2x + 395.3$ was found for male *P. xylostella* average dark phase activity counts against length of dark phase in h. A significant trend was also found for females of $R^2 = 0.55$, $Y = 23.6x + 192.9$. These slopes are not significantly different, however female results have lower values in line with lower recorded average activity. These slopes show decreasing amount of activity expressed during shortening dark phase lengths, however, rates of activity during dark phase remains non significantly different between most conditions. The slope of the simple linear regression lines is notably different from that of a constant dark phase activity rate and constant total dark phase activity. As a proportion these results shows for every h loss from

dark phase period length ~2.41% of activity transfers into light phase, which is significantly different than if such was directly proportional to dark phase length. Interpolated values from the trend line show ~62.8% dark phase activity at 3h of dark phase, ~60.4 at 2h, ~58.0% at 1h, intercepting the y-axis at 55.6%. There is consistency between the % of activity occurring during the dark phase of 12/12 and 6/6/6/6 conditions, where both have 12h of dark phase but split into 2 for 6/6/6/6. These two conditions were combined for 12h of dark phase results in Fig. 3.10.

Fig. 3.11 shows how both male and female adult *P. xylostella* distribute their activity paired between (s) light and (s) dark phases compared to how *P. xylostella* in D/D and 6/6/6/6 spread there's between each 12h period.

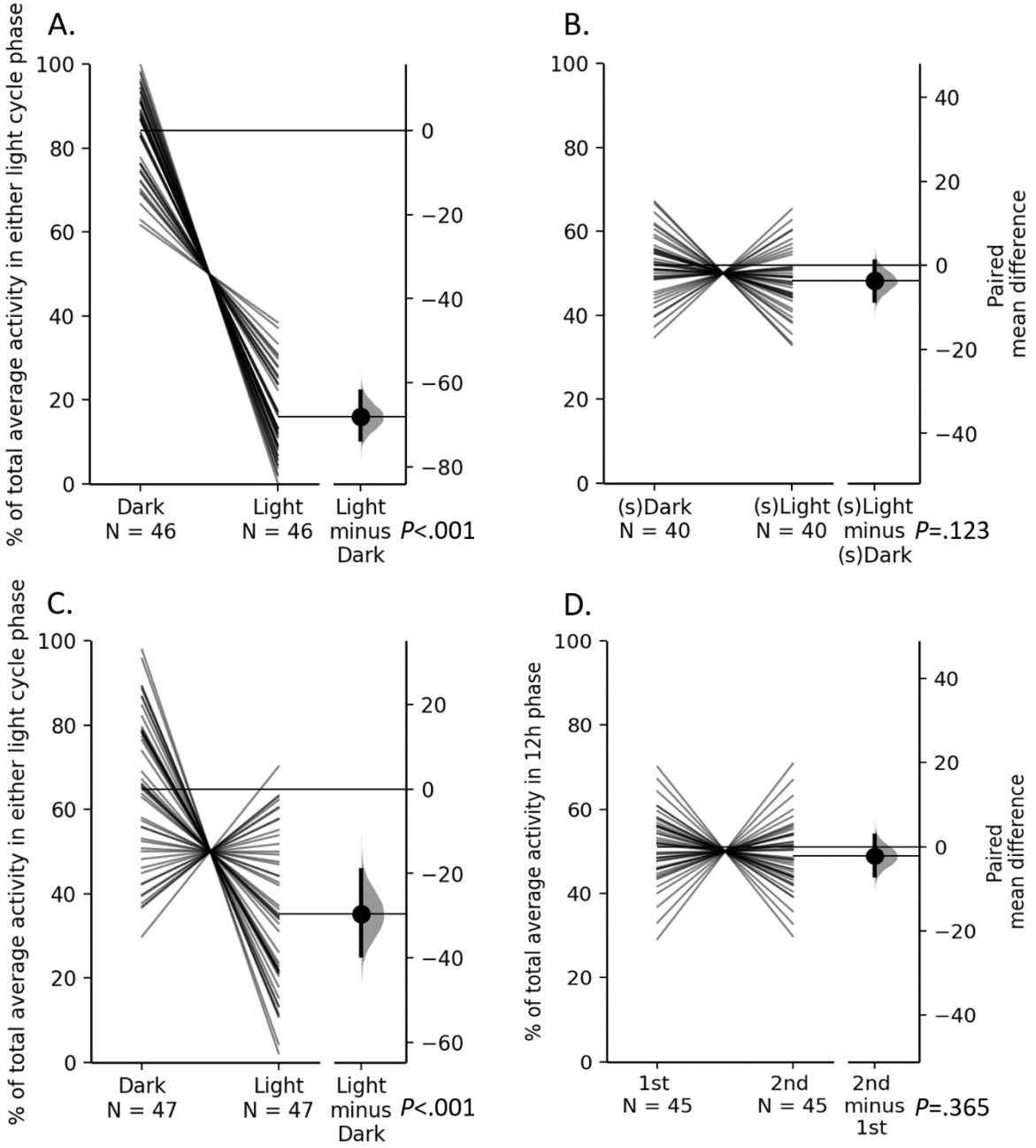


Figure 3.11 Adult *P. xylostella* Tufte slopegraph comparisons.

The majority of adult *P. xylostella* moths express most of their activity during dark phases of light cycle conditions, with roughly even split of activity between 1st and 2nd 12h phases of 6/6/6/6 and D/D conditions. Tufte slopegraphs show A) proportion of total activity collected from male and female adult *P. xylostella* in a 12/12 light cycle split between dark and light phase activity paired to their respective moth, B) proportion of total activity in D/D (s) dark and (s) light phases, C) proportion of total activity in 20/4 dark and light phases, D) proportion of total activity in 6/6/6/6 split between the first and second 12h periods. N indicates number of moth repeats 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.

The findings from Fig. 3.6 and 3.7 show some significant changes in activity, but total activity and activity rate remains relatively consistent and though activity occurring in dark phase trends down, as shown in Fig. 3.10, the activity profiles show similar rhythmic profiles. The 12/12 condition in Fig. 3.11A shows how out of a mix of 46 male and female moths tested, none showed less than 60% activity in a single phase, with significantly more activity in the dark phase. When light cycles are lost in D/D, shown in Fig. 3.11B, a 12h phase preference is lost with neither s dark nor s light phase having significantly more activity overall. In Fig. 3.7C the range of only males in 20/4 dark phase activity % is not significantly different from D/D. Fig. 3.11C of males and females in 20/4 shows further how though the average is still above 50%, adult *P. xylostella* in this condition cannot always maintain dark phase focused activity, with a number of moths falling below 50% activity in dark phase, being the only light cycle condition to have moths that do so. When the 12h phases are split in two, 6h of dark, 6h of light, as in the 6/6/6/6 condition, 12h phase preference is lost, with there being no significant difference between total activity in each 12h phase as shown in Fig. 3.11D, with the 1st 12h phase being entrained/ s dark phase before condition setup. Only 5/45 moths have >60% of their activity falling into one 12h phase.

Maintenance of rhythms into D/D is also not shown using locomotor assays held at a constant 20°C, as shown in Fig. 3.12, 3.13 and Table 3.6. 12/12 consists of all surviving moths in this condition, cutting of data analysis before D/D condition start, with D/D containing only the moths that survived to end of data collection period shown in Fig.3.12A with separated data for D/D period.

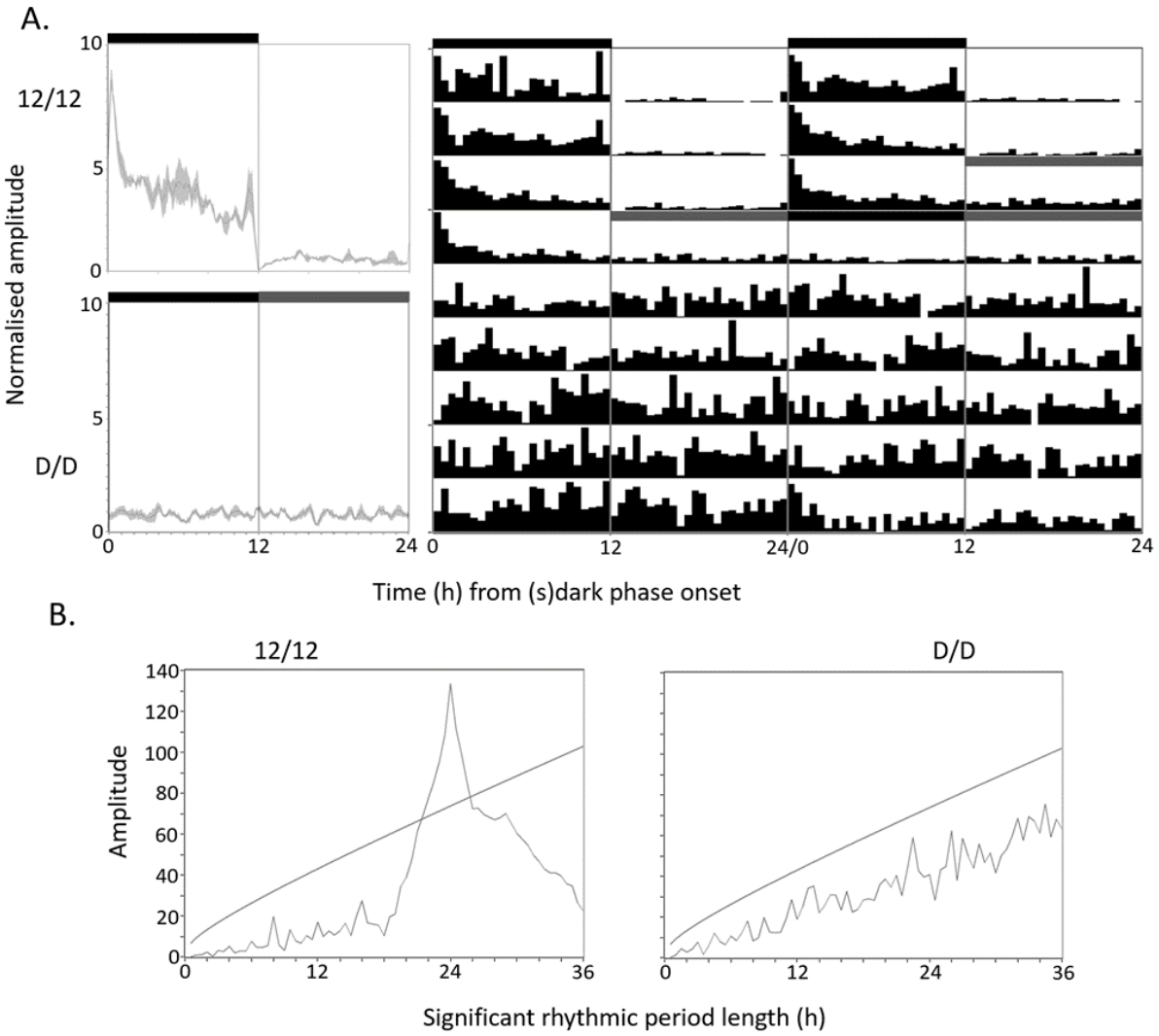


Figure 3.12 Adult male *P. xylostella* in 20°C under 12/12 and D/D light conditions.

Adult male *P. xylostella* show comparable rhythmic profile and features under 20°C constant temperatures in 12/12 and D/D light conditions compared to 17°C.

A. Average activity profiles (Left) show the average activity over 24hrs of the 3-day average activity in 12/12 and 6 day average activity in D/D. Time is plotted along x-axis in hours (h), starting at dark phase and subjective (s) dark phase onset with normalized amplitude along y-axis. The D/D average starts from first complete day in D/D. Central line shows the average activity with the shaded area showing SEM. The black bar along the top of individual graphs shows dark phase with shaded bars showing s light phase for D/D. Double plotted actograms (Right) show cross over of data between conditions, showing average activity of moths normalized against the

next day's activity in 30-minute bins of counts with transition to D/D after 4 complete days in 12/12.

B. Average periodograms showing significant rhythm period lengths found from the data in 12/12 (left) and D/D (right) conditions with period length along x-axis and amplitude of significant rhythm period on along y-axis. Pale grey line shows significance threshold for period lengths and dark grey ling showing amplitude of rhythmic period likelihood from data.

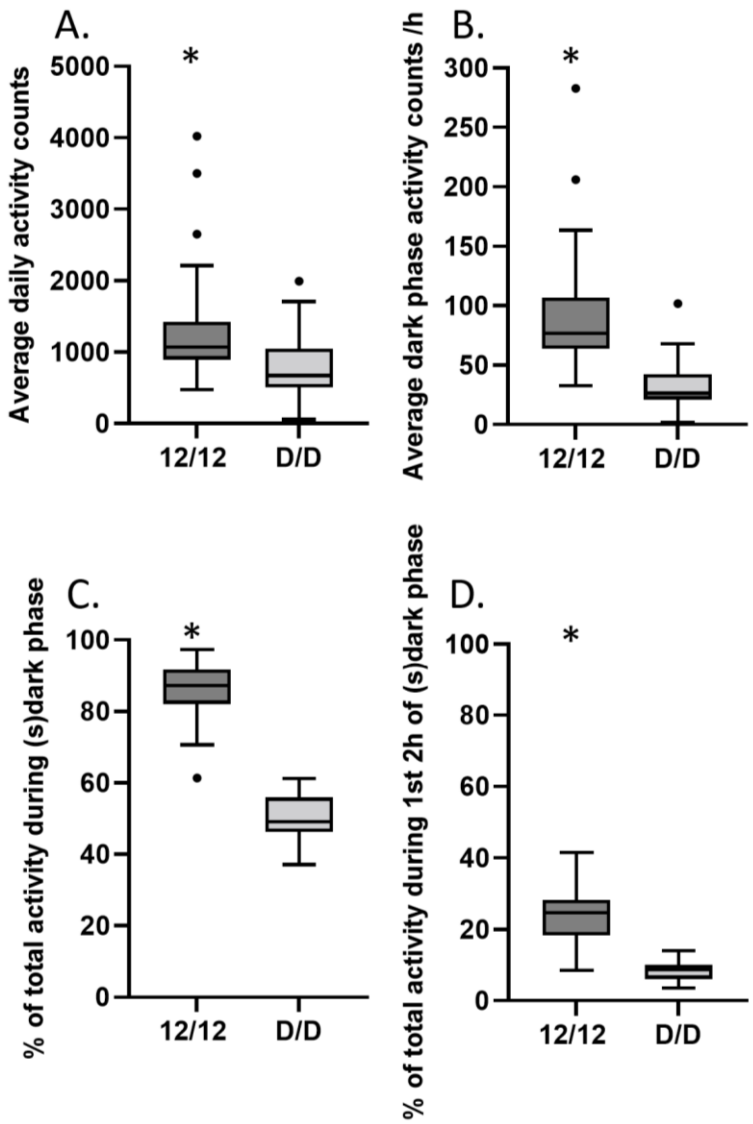


Figure 3.13 Adult male *P. xylostella* behavioural analysis between 12/12 and D/D at 20°C.

Adult *P. xylostella* display significant differences between the average amounts activity between 12/12 and D/D conditions. The box and whisker plots demonstrate shifts in features of locomotor activity over conditions, showing A. total average activity over 24 h of adult *P. xylostella* in varying light cycle conditions, B. total dark phase average activity divided by dark phase period in h, C. average % of total activity occurring during (s) dark phase, D. average % of total activity occurring during first 2h of (s) dark phase. Data points outside Tukey range (1.5XIQR (Interquartile range)) are shown. Mann-Whitney U test results show significant differences from each respective data set through *.

Table 3.7 Adult male *P. xylostella* at 20°C under 12/12 and D/D periodogram data.

Data from Fig. 3.11 ±SEM. % of moths that had significant periodogram rhythms with average period length and RRP of such. (n) number of repeats, (h) hours. RRP calculated using ratio between periodogram dominant period likelihood amplitude and significance threshold for h length of specified period.

Condition (n)	% rhythmic	Period length (h)	RRP
12/12 (37)	57	24.3 ±0.5	1.22 ±.04
D/D (18)	0	n/a	n/a

Data is consistent with previous findings with significant differences found in Fig. 3.13A and B due to activity being highest at beginning of assay set up.

3.3.4 qPCR clock gene analysis

The rhythmic profile of *tim* and *per* expression both closely match each other when analysed against *Ef1α* (*Elongation factor 1 α*) using qPCR (Quantitative polymerase chain reaction) as shown in Fig. 3.14, with no time point significantly different between the two and no significantly different rhythmic features shown in Table 3.8.

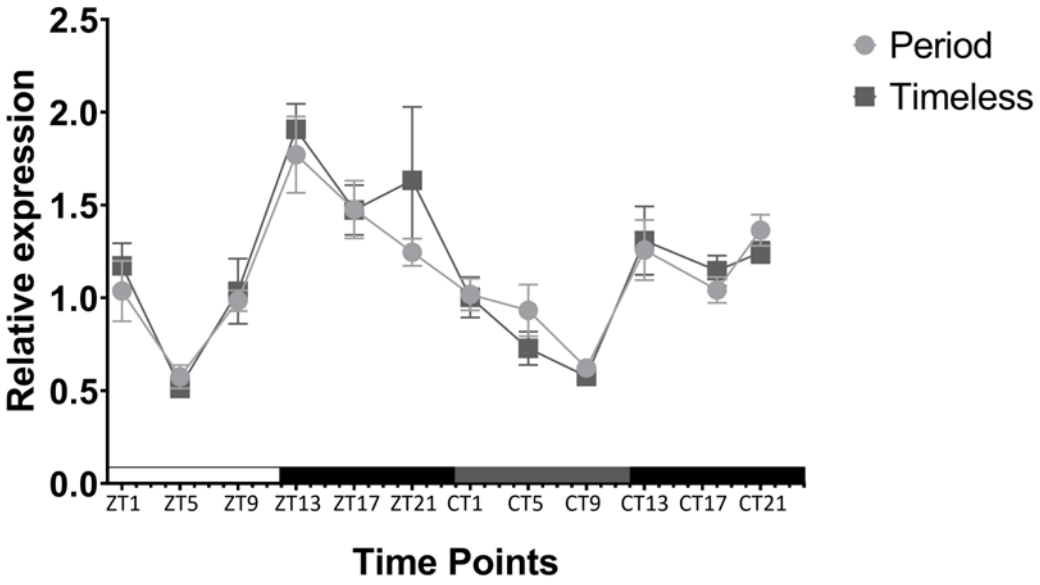


Figure 3.14 Adult male *P. xylostella* qPCR relative expression of *per* and *tim.*

Adult *P. xylostella* rhythmic mRNA expression levels of *per* and *tim* maintain rhythmicity in D/D. Line graph with SEM showing relative expression of clock genes *per* and *tim* over time relative to the *Ef1α* housekeeping gene, with 12/12 L/D zeitgeber time denoted by ZT time points and a grey bar for circadian dark phase and CT time points in D/D.

Table 3.8 CircaCompare results of adult male *P. xylostella* qPCR relative expression of *per* and *tim*

CircaCompare fitted cosine features for each gene. Phase peak shown as ZT time points and for CT. Rhythm *P* equates to the *P* value of data's fit to cosine curves, 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 ZT time points and respective CT time points where the peak of fitted curve occurs.

Condition	Rhythm P	Mesor	Amplitude	Phase peak
per	3.19^{-7}	1.11	0.34	16.86
tim	3.89^{-7}	1.14	0.42	17.12

The cosine curve oscillations of *tim* and *per* are not significantly different from each other, shown in Suppl. Fig 3, however, both *per* and *tim,* when comparing their own 12/12 to D/D

expression profiles have significant differences as shown in Table 3.9 and 3.10. Relative expression levels at ZT5, 9 and 17 are significantly different to their respective CT time points in *per* expression with ZT9 and 13 significantly different for *tim*. Shown in Suppl. Fig. 2, both 12/12 and D/D expression profiles are rhythmic but the amplitudes of such are lower in D/D with *per* showing significantly lower rhythmic amplitude while *tim* has a significantly lower mesor in D/D. *per* also shows a significantly different phase between 12/12 and D/D. However, when comparing *per* and *tim* expression profiles directly, shown in Suppl. Fig. 3 CircaCompare shows no significant differences in their rhythmic profile features. Table 3.10 shows that most the variation of the time course comes from changes in time, however there is also significant interaction between changes in time and the L/D and D/D condition for *per.*

Table 3.9 ANOVA results of adult male *P. xylostella per* and *tim* expression.

ANOVA tests of 24h L/D and L/L rhythms and comparisons between L/D and L/L day time points. Tukey's multiple comparison test shows significant differences between specific time points.

ANOVA	*Per* L/D	*Per* D/D	*Tim* L/D	*Tim* D/D
P value	*P* <0.0001	*P* <0.001	*P* <0.001	*P* <0.0001
Time points	Sig	Sig	Sig	Sig
ZT1/CT1	ab	ab	ab	ab
ZT5/CT5	b	b	ab	ac
ZT9/CT9	bc	bc	b	a
ZT13/CT13	d	a	a	b
ZT17/CT17	acd	ac	ab	bc
ZT21/CT21	acd	ac	a	b

Table 3.10 2-way ANOVA results of adult male *P. xylostella per* and *tim* expression.

2-way ANOVA results also shown for combination between effects of time over 24h day and L/D or L/L conditions, in addition to whether there is any significant interaction between the two variables, that account for variation in relative.

2-way ANOVA	*Per* L/D vs D/D %	*P* value	*Tim* L/D vs D/D %	*P* value
Condition	2.848	*P* <0.01	7.330	*P* <0.01
Time	42.38	*P* <0.0001	41.86	*P* <0.0001
Interaction	14.18	*P* <0.01	5.789	*P* >0.05

3.4 Discussion

3.4.1 Effects of temperature cycles on adult *P. xylostella*

Through the data shown in Fig. 3.3, adult *P. xylostella* show potential temperature responses in locomotor activity, however, the increases in activity that follow temperature fluctuation in light phase in both April and June conditions don't respond directly to increases in temperature. Activity continues as temperature decreases, as well as showing little to no activity around peak temperature in June conditions. The activity around peak temperature in June conditions may be a sign of an escape responses due to changing environmental temperatures rather than increased activity due to the heating of flight muscles as shown in previous research (Kammer, 1965, Heinrich and Mommsen, 1985, Heinrich, 2007). The drop in activity at peak temperature may also occur as the *P. xylostella* population is typically reared at 20°C, so may be more likely to reduce activity in light phase at this temperature due to habituation, with moths not reliant on increased temperatures for flight activity (Thorpe, 1943, Heinrich, 2007, Ma and Ma, 2012, Hackl

and Robertson, 2019). In April conditions the peak temperature occurred over a longer period than in June and at a lower temperature (~16°C), likely causing the differences in temperature response seen. The changes in light conditions however elicit the greatest response in activity compared to temperature, which has previously been shown to be a weaker entrainment factor in comparison to light (Syrova, Sauman and Giebbultowicz, 2003). The transitionary phases between light and dark phase have minimal effect, instead the mostly complete absent or presence of light causing a prominent activity peak for the former or cut off in activity for the latter in adult *P. xylostella.*

3.4.2 Light repression of adult *P. xylostella* activity

The extent of light repression of activity seen between dark and light phases is shown across many results, demonstrated in Fig. 3.4B where average L/L activity is barely apparent against moths in light cycle conditions and D/D. The results in this chapter demonstrate that *P. xylostella* is very sensitive to the lights used in experimental set ups and therefore likely sensitive to ALAN (Macgregor et al., 2014). During transition phases of the April and June mimic conditions in Fig. 3.3A activity profiles, as lights begin to ramp up in intensity activity quickly and consistently drops, though in June conditions is maintained after temperatures begin changing. During the transition to the dark phase, it is also only after complete lights off the characteristic dark phase onset peak occurs, which is also shown across light cycle conditions in Fig. 3.4A. Results from light phase transition phases, in addition to the findings from the set up in Fig. 3.5, confirm that sensitivity to light and other findings in this chapter are not solely due to the main locomotor activity assay set up. In the light cycle locomotor assay set up used, test tube isolated moths are subjected to sudden onset of artificial lights which may have caused light-startle responses as seen in other studies (Truman, 1974, Fenckova et al., 2019, Varnon and Adams, 2021). The semi-field and population set ups were important to analyse as they more closely fit

natural environmental conditions with one having both temperature and light cycles and one with *P. xylostella* mixed-sex groups able to fly freely and interact with each other. These forms of semi-natural set ups are important to confirm more controlled lab results findings and expose any secondary behavioural features, which have been shown to appear when further abiotic and abiotic factors are included in experiments (Kannan et al., 2012, Nagy et al., 2018, Shaw, Fountain and Wijnen, 2019). Adult *P. xylostella* activity rhythms showed clear nocturnally focused activity across all locomotor set ups and light cycle conditions. The dark phase focused activity of *P. xylostella* adults matches many previously studied nocturnal moths demonstrating avoidance of light phases (Kawahara et al., 2018). The locomotor assays produced a distinct dark phase onset peak in activity with an increasing proportion of total activity being part of such as dark phase length decreased. This is similar to research that has shown *P. xylostella* to express a large proportion of their behaviour in the first h of dark phase with over 70% of mating and oviposition taking place before the end of the first 4h of such in 16/8 conditions (Pivnick et al., 1990). The lack of maintenance of these rhythms into constant conditions however is more notable as related species have been shown to possess rhythmic circadian clock gene expression

3.4.3 Relative expression of adult *P. xylostella* circadian clock genes

Our findings that adult *P. xylostella* have rhythmic cycling of *per* and *tim* matches papers that commonly show similar rhythmic expression profiles with lower levels of expression in light phase compared to dark phase in moths (Merlin, Gegear and Reppert, 2009, Kobelkova et al., 2015, Yan et al., 2017, Zhang et al., 2021). Under D/D conditions however, a study showed *tim* transcripts and TIM to be lowered compared to dark phase in L/D conditions in *D. plexippus* (Merlin, Gegear and Reppert, 2009), while another showed clear maintenance of both *tim* and *per* expression rhythms in *Helicoverpa armigera* (Yan et al., 2017). It can therefore be unclear between species how the expression of clock genes

is maintained and translated, furthering the idea of a need for species-specific profiles which this chapter now provides for *P. xylostella* adults. Under D/D, the expression profiles in Fig. 3.9 show both *per* and *tim* had lower amplitude in relative expression values compared to L/D, with significantly reduced rhythmic amplitude for *per* expression. This maintenance of rhythmicity but with reduced amplitudes, shows results somewhat between the two previously referenced papers, similar to a previous study on *Ephestia kuehniella* (Kobelkova et al., 2015), and show that *P. xylostella* adult clocks may have reduced rhythmicity following the loss of external stimuli.

3.4.4 *P. xylostella* adult locomotor activity lacks evidence of circadian clock control

Though the results from qPCR analysis showed rhythmic cycling of circadian clock genes, there is minimal evidence of circadian control of *P. xylostella* locomotor activity provided by the locomotor data. Though loss of adult *P. xylostella* activity rhythms was shown in L/L conditions, it is the loss of rhythms under D/D conditions that is more unexpected. D/D conditions often allow for endogenous free-running rhythms to become apparent like that seen in *D. melanogaster* (Shindey et al., 2016, Dubowy and Sehgal, 2017). Though many studied lepidopteran species express rhythms in D/D there are some that also show loss of rhythms, including *Hyles lineata* (Broadhead et al., 2017) and female *P. interpunctella* (Zavodska et al., 2012), these findings, as with this chapters adult *P. xylostella* results, do not show evidence of active circadian control of the tested behaviours. The findings from *P. xylostella* in caged populations show activity proximal to a food sauce more specifically than general activity like those in the other locomotor set ups, and so provides further evidence that adult *P. xylostella* does not maintain rhythmic activity endogenously into D/D. A recent paper has corroborated these findings in *P. xylostella*, including dark phase activity peaking, yet demonstrates a form of temperature gated D/D rhythmicity in male *P. xylostella,* becoming rhythmic at lower temperatures

(Wang, Yang and Chen, 2021). Through comparisons between male and female *P. xylostella* adults presented in this chapter we also showed lower levels of rhythmicity in females which is supported by Wang et al. 2021, in addition we also found significantly reduced survival and average activity in a number of light cycle set ups. A study on hawkmoths also showed significantly lower female activity compared to males in certain conditions (Broadhead et al., 2017). It has been found that female *Cnaphalcrocis medinalis* eclose earlier than males (Guo et al., 2019) along with certain other lepidopteran species (Degen et al., 2015), therefore tested males and females may have age related differences.

There appears to be no phase anticipation throughout our results, where there is no notable increase in light phase activity in adult *P. xylostella* prior to dark phase onset, which would otherwise be a sign of active circadian control of the recorded behaviour (Stoleru et al., 2004). Another key piece of evidence further to absent D/D rhythmicity, to show *P. xylostella* lacks circadian control of locomotor activity, is the lack of circadian modulation that should be apparent via the comparison of 12h phases. It is also expected for a s circadian dark and light phase to be maintained, however, in 6/6/6/6 conditions there is no significant selection for one 12h period to be the s dark phase over the other, as shown in Fig. 3.4A and Fig. 3.7D, where the results show adult *P. xylostella* would otherwise focus ~80% of activity. This circadian modulation, which could be expected in insects with active circadian clocks (Wheeler et al. 1993), is important due to anticipatory regulation of physiology, as it has been shown in these s rhythmic periods that many physiological processes from general metabolism to formation of memory can be modulated depending on what perceived phase the organism is in (Lyons et al. 2005, Dubowy and Sehgal, 2017). Instead of adult *P. xylostella* 'choosing' one 12h cycle over another as the s dark phase activity period, they appear to react directly to the 6/6 12h L/D cycle, this is backed up by the arrhythmic results from the constant conditions experiments showing no maintenance of s phases. An experiment on *Spodoptera frugiperda* adults shows similar direct reaction

to light schedule changes through a 6h phase advance where *S. frugiperda* immediately maintains the advanced 24h cycle (Miranda-Anaya, Guevara-Fefer and Garcia-Rivera, 2002).

3.4.5 Homeostatic regulation of *P. xylostella* locomotor activity

P. xylostella may lack recordable circadian control of locomotor activity due to the behaviour the used assays are capturing being primarily regulated by homeostatic pathways. Homeostatic processes are endogenous controls to maintain balance within an organism, reacting to both internal and environmental cues, they may inform physiological systems of stressors such as temperature changes or caloric intake or deficit leading to the regulation of behaviours (Drengstig et al. 2012, Liu and Kanoski, 2018, Ruoff et al., 2019). *P. xylostella*, while expressing >80% of their locomotor activity during the dark phase of 12/12 conditions, maintains non-significantly different total average activity as dark phase period is reduced. *P. xylostella* appears to maintain this via a maximal rate of activity during dark phase conditions with dark phase activity rate lowering above 12h of dark phase. Due to this limit in homeostatic modulation of activity rate, more activity appears to bleed into light phase as dark phase period decreases, with Fig. 3.6 estimating at a rate of ~2.4% of total activity per h lost. As total average activity is not significantly lower at 20/4 conditions compared to 12/12, while only ~64% of activity occurs during the dark phase, it shows that maintenance of a regular amount of activity overcomes light repression to a certain extent. Other studies have found compensation in distribution of activity to maintain overall constant rates in a variety of animal species (Reynolds, Yeomans and Timmins, 1986, Heurich et al., 2014), along with the homeostatic regulation of activity through maintenance of sleep (Cirelli and Bushey, 2008, Deboer, 2018). Maintenance of either constant total dark phase activity or constant dark phase activity rate in *P. xylostella*

is also not apparent, shown in Fig. 3.3, with limiting of total activity seen in conditions with longer dark phase periods and limiting of dark phase activity rate seen in shorter.

3.4.6 Why may adult *P. xylostella* locomotor activity be under homeostatic regulation

It may be beneficial for adult *P. xylostella* to express as much activity as it can during dark phase periods to avoid activity during light phase and related potential predation, where previous research has too shown *P. xylostella* to focus the majority of feeding, mating and oviposition behaviours in the dark phase (Pivnick et al., 1990, Lee, Lee and Boo, 2005). Therefore, activity is highly expressed as soon as a certain level of darkness is achieved that *P. xylostella* has been selected for to exploit. This may also relate to migratory behaviours where *P. xylostella* has been shown to migrate overnight (Chapman et al., 2002, Wainwright et al., 2020), therefore the provision of dark phase conditions in experimental set ups may trigger some forms of dispersal activity. Reacting directly to light conditions rather than photoperiodic entrainment may also be advantageous due to the scale in shifts of such during rapid *P. xylostella* migration (Talekar and Shelton, 1993, Coulson et al., 2002, Chapman et al., 2002). These behaviours may also react with the presence of conspecific or plant host volatiles where the isolation from sex pheromones and signals from potential plant hosts may affect varying behavioural responses such as those recorded in this chapter (Pivnick et al., 1990, Campos, Schoereder and Sperber, 2004, Campos, 2008).

The total locomotor activity expressed by adult *P. xylostella* in this chapter may be determined by energy expenditure. *P. xylostella* migrate predominantly using air currents (Chapman et al., 2002), therefore locomotor activity is not directly proportional to the distance travelled during migration events. Reduced growth rates may correlate with higher energy expenditure in lepidopterans (Hof and Martin, 1989) and there may be homeostatic

limits on energy intensive behaviours to preserve energy reserves (Liu and Kanoski, 2018). Energy may be conserved for use to migrate to sites with plant or *P. xylostella* volatile signals which is potentially lacking in the isolated assays used for light cycle analysis in this chapter, therefore when increased h of dark phase periods were provided there was only a limited increase in activity due to lack of *P. xylostella* sex and plant pheromones to stimulate further dark phase behaviour beyond the limits recorded in this chapter. Further to this, in the results from caged mixed-sex population experiments measuring activity proximal to food source, there was significantly more counts over 24h in D/D compared to 12/12, differing from the individual locomotor assays that showed no significant difference. Sex and plant pheromones can have many effects on organisms' activity including those recorded in lepidopterans (Miranda-Anaya, Guevara-Fefer and Garcia-Rivera, 2002, Crespo, Goller and Vickers, 2012). *P. xylostella* specifically shows clear modulation of behaviour and sex interactions depending on the presence of plant volatiles with ~90% of females becoming sexually active in the presence of plant hosts while only 27% when a plant host was absent (Pivnick et al., 1990). It has been previously shown that the life history traits of *P. xylostella* can be significantly shifted depending on the host plants *P. xylostella* is exposed to (Rossbach, Lohr and Vidal, 2007, Golizadeh et al., 2009), therefore understanding *P. xylostella* interactions with plant volatile signals will be important for understanding how the activity rhythms of adult *P. xylostella* may be influenced in natural environments.

3.4.7 Conclusion

This chapter outlines adult *P. xylostella* activity and what may be controlling such. Temperature may have some effects on *P. xylostella* activity and control, however, it is changes in light conditions that showed the largest responses in activity. Adult *P. xylostella* showed high sensitivity to light and therefore potentially ALAN, directly responding to its

presence or absence in locomotor assays. Activity rhythms were found to be nocturnal with distinct dark phase onset peaks in activity. The expression of *per* and *tim* was also found to be rhythmic, demonstrating evidence of an active molecular circadian clock. However, there is minimal evidence of circadian control of locomotor activity with a loss of activity rhythms in D/D, no significant circadian modulation of such and no anticipatory activity. The lack of apparent circadian control in recorded behaviour may be due to regulation of activity via homeostatic pathways. Apparent limits in total activity and activity rate were found in addition to the maintenance of a typical amount of activity via the increased expression of activity during extended light phases. These findings are likely determined by *P. xylostella* life history traits and adaptations along with interactions with *P. xylostella* and host plant volatiles, requiring further research into how interactions between *P. xylostella* and host plants may affect behaviours. The demonstrated results may be useful for aiding the timing of IPM strategies used against adult *P. xylostella* as well as for understanding what may affect and control adult *P. xylostella* activity rhythms.

Chapter 4 Determinants of Diel Herbivory Rhythms of Diamondback moth (*Plutella xylostella*) larvae.

4.1 Abstract

Plutella xylostella is a global pest of cruciferous crops with damage caused likely to increase with climate change and an expanding host range. *P. xylostella* causes ~$5 billion of damage annually, being able to outcompete pesticides and natural plant defences. Glucosinolate sulfatase genes unique to *P. xylostella* disarm the mustard oil bomb, a defence unique to Brassicaceae, giving *P. xylostella* an advantage over non-specialised insect herbivores. Both pest and host plants show circadian regulation of numerous physiological processes and interactions between the regulation of plant defences and *P. xylostella* may be important in determining herbivory. Understanding these interactions may help identify ways to exploit such to improve both crop defences and integrated pest management. We carried out *P. xylostella* larval feeding assays, combining light/dark experiments with deepening constant light pre-exposure and in and out-of-phase entrainment with host plant leaf tissues. Results showed maintenance of *P. xylostella* rhythmic feeding into constant light conditions with clear peak feeding periods. Changing the circadian entrainment of host plants significantly altered these rhythms, demonstrating interactions with host plant signals. Larval weight gain results somewhat matched these findings but were highly variable, likely due to rhythms in defecation. The cyclic expression of key circadian clock genes *period* and *timeless* was also shown in *P. xylostella* larvae, with maintenance of both these and glucosinolate sulfatase genes into constant light conditions and when larvae were fed out-of-phase leaf tissue, showing endogenous circadian rhythms in *P. xylostella* larvae. Using these findings with mutant *A. thaliana* feeding assays demonstrated both interactions between *P. xylostella* larvae and circadian clock and jasmonic acid pathways in host plants. These results show potentially

exploitable rhythms in *P. xylostella* feeding and detoxification genes for improving integrated pest management strategies via the precise timing of treatments and or enhancement of natural plant defences.

4.2 Introduction

The increasing severity of pest species infesting crops (Deutsh et al., 2018), having the potential to cause over $540 billion of losses annually (Paini et al., 2016), is one of the preeminent hurdles modern agriculture has to overcome while simultaneously reducing harm to the natural environment (Pimentel and Edwards, 1982, RCEP, 2005, Mahmood et al., 2015) and doubling yield to feed an exponentially increasing human population (Ray et al., 2013). Losses caused by pests are underestimated (Bradshaw et al., 2016) and likely to multiply due to climate change (Early et al., 2016, Paini et al., 2016, Savary et al., 2019, Skendzic et al., 2021), increasing the urgency for novel research to uncover new avenues of pest control. The widespread rise of pesticide resistance and the increasing interconnectivity of a globalised food market is straining classical control techniques and fuelling the move to 'precision agriculture' (Barnes et al., 2019). The cruciferous agricultural pest *P. xylostella* (the Diamondback moth) is arguably the world's most economically damaging Lepidopteran, having achieved a cosmopolitan distribution (Talekar and Shelton, 1993), affecting up to 92% of brassica farmers in certain regions (Uthamasamy et al., 2011). The annual losses caused by *P. xylostella* globally has risen from ~$1 billion in the 1990s (Talekar and Shelton, 1993) to ~$5 billion (Zalucki et al. 2012, Furlong, Wright and Dosdall, 2013). These global losses are facilitated by *P. xylostella's* ability to migrate, for which it is extremely proficient at, able to cross continents over a season and near 1000km in ~48 hours (h) (Chapman et al., 2002, Coulson et al., 2002), having spread to all but the coldest latitudes in a seasonally expanding range (Furlong, Wright and Dosdall, 2013). The consensus is currently unclear over *P. xylostella*

origin, potentially increasing the difficulty of uncovering natural control mechanisms for pest populations, but is split between South Africa (Kfir, R. 1998), South China (Liu *et al*. 2000), Europe (Hardy,1938, Harcourt, 1954, 1957) or most recently South America (You et al., 2020). The precise origin region may be more ambiguous than any singular site however due to *P. xylostella* migratory behaviour (Campos, Schoereder and DeSouza, 2006). *P. xylostella* migrates via air currents (Talekar and Shelton, 1993), subsequently seeking out brassica crops (Shelton, 2004, Wei et al., 2013), sensing a unique secondary metabolite to Brassicaceae called glucosinolate (Feeny, 1977), for which *P. xylostella* is strongly attracted to (Moldrup et al., 2012, Badenes-Perez, Gersehnzon and Heckel, 2014, Robin et al., 2017). Glucosinolate likely became an attractant for *P. xylostella* due to having a competitive advantage over other insect herbivores feeding on brassicas, as *P. xylostella* possesses a unique specialised protein called glucosinolate sulfatase (GSS). These enzymes break down glucosinolates to prevent the build of toxic herbivory deterrents through a process unique to Brassicaceae called the MOB (Mustard oil bomb) (Ratzka et al., 2002, Kumar, 2017). Control methods are being implemented and researched for controlling *P. xylostella* pest populations ranging from more traditional control practices such as intercropping (Buranday and Raros, 1975, Sivapragasam, Tee and Ruwaida, 1982) to germline transformation using minigene constructs aiding genetic modification of *P. xylostella* for SIT (Sterile insect technique) releases (Jin et al., 2013, Bolton et al. 2019). These techniques are being implemented as chemical pesticide treatments are facing repeated failures, in addition to the environmental damage they cause (Wilson and Tisdell, 2000, Aydinalp and Porca, 2004, RCEP, 2005, Jepson et al., 2014, Mahmood et al., 2016), due to *P. xylostella*'s suit of advantageous traits for gaining pesticide resistances (Shelton et al., 1993, Talekar and Shelton, 1993, Heckel DG, 1999, You et al., 2013, Xia et al., 2018, Jaleel et al., 2020), being known as the first pest to become resistant to DDT (Dichlorodiphenyltrichloroethane) and *Bt*T (*Bacillus*

thuringiensis toxin) (Ankersmit, 1953, Tabashnik et al., 1990, Tabashnik et al., 1997). Such treatments, in addition to a range of less environmental damaging and species-specific control methods employed by IPM (Integrated pest management), may be found to be more effective with the precise timing of applications, with the study of chronotoxicology dating back half a century (Beck, 1963, Shipp and Ottom, 1976, Miller et al., 2002, Mitchell, 2002, Tkadlec and Gattermann, 2008, Ikegawa et al., 2021, Wang et al., 2021, Bull and Gomulkiewicz, 2022). Therefore, the enhancement of IPM strategies through novel pathways may be useful at improving the control of *P. xylostella* pest populations, such as with the exploitation of both pest and host-plant circadian rhythms.

Circadian rhythms can be maintained without the need for external stimuli but can be entrained by such along with varieties of chemical and physical cues (Reppert and Weaver, 2002, Gardner et al., 2006, Fuchikawa et al., 2016, Zheng et al., 2021), being harnessed by life to keep in time with the ~24h daily cycles of the planet (Pittendrigh, 1993, Foster and Kreitzman, 2005). Circadian clocks can be used to maintain and control rhythmic activity and confer widespread daily rhythmicity to the physiology and behaviour of organisms, maintained via a series of Transcription and translation feedback loops (TTFL) (Hurley, Loros and Dunlap, 2016, Dunlap and Loros, 2017). The core components of the circadian clock have been demonstrated in a multitude of model organisms including *Arabidopsis thaliana* (McClung, 2006), *Mus musculus* (Chang and Reppert, 2001), *Drosophila melanogaster* (Rosato, Tauber and Kyriacou, 2006) and *Danaus plexippus*, a Lepidopteran (Reppert, 2007, Kyriacou, 2009, Brady et al., 2021). The plant and animal circadian clocks differ greatly but both share the key principles of the TTFL model. The circadian clock mechanism of *D. plexippus* has similarities however to findings from both the arthropod and mammal models. *per* (*period*) and *tim* (*timeless*) genes were among the first discovered animal circadian clock genes and are shown to make up part of the core cyclic part of molecular circadian clocks (Rosato, Tauber and Kyriacou, 2006). PER and TIM

produce a heterodimer that inhibits their own gene expression by inhibiting an additional cyclic pathway involving *Clk* (*Clock*) and *cyc* (*cycle*) shown in *D. melanogaster* and *Clk* and *Bmal1* (*Brain and muscle ARNT-like 1*) in mammals and Lepidoptera. A key difference between the *D. melanogaster* model and the lepidopteran model is the presence of mammalian-like cryptochrome (mCRY) with PER in the nucleus (TIM fulfils this role in *D. melanogaster*) for inhibiting the CLK-BMAL1 complex. Lepidopteran TIM doesn't enter the nucleus however still interacts with light-sensitive *Drosophila*-like (d)CRY pathway, similar to *D. melanogaster* (Zeng et al., 1996, Hardin, 2005, Zhu et al., 2006, Yuan et al., 2007, Kyriacou, 2009). The plant clock has a novel suite of translation and transcription factors with both blue and red light associated receptor proteins having a key role in the core molecular clock. While the clock differs in its components from that of animal clock mechanisms, using *cca1* (*circadian clock associated 1*), *lhy (late elongated hypocotyl)* and *toc1 (timing of CAB expression 1)* as the core molecular clock, the use of interconnected feedback loops of gene expression, phosphorylation and degradation resembles that of animals, preserving the role of TTFL models (Gardner et al., 2006, McClung, 2006). The plant circadian clock is heavily involved in the regulation of virtually all aspects of plant biology, likely due the adaptations necessary for sessile organisms to adapt to the environment around them (Inoue, Araki and Endo, 2018, Jimenez, Sevilla and Marti, 2021). An estimated 35% of the total plant transcriptome is under circadian control (Michael and McClung, 2003) with that proportion increasing to ~40% for wounding associated genes (Walley et al., 2007), showing circadian rhythms have a disproportionately large effect on plant defences, with many tissue-specific metabolite rhythms sensitive to herbivore attack (Kim et al., 2011). Plant secondary metabolites important in growth and defence such as jasmonate and glucosinolate are produced rhythmically and in response to both biotic and abiotic stressors (McClung, 2006, Goodspeed et al., 2013a, Goodspeed et al., 2013b). Circadian clocks can have wide

reaching control over many aspects of animal physiology, particularly with feeding, immune responses and the anticipation of threats (Kim et al., 2011, Scheiermann, Kunisaki and Frenette, 2013, Curtis et al., 2014). Numerous behaviours and tissue activity can be rhythmic with tissue-specific circadian clocks found in many organs, including immune cells (Chaix, Zarrinpar and Panda, 2016, Aoyama and Shibata, 2017, Serin and Acar-Tek, 2019). The rhythmic production of secondary metabolites and herbivory deterrents appears to be reliant on the function of the plant molecular circadian clock (Joo et al., 2018, Zhang, Bo and Wang, 2019, Valim et al., 2020). Genes have also been identified along specific JA biosynthesis pathways such as *aos* (*allene oxide synthase*)*, opr3* (*oxophytodienoate-reductase 3*) and signalling genes such as *coi1* (*coronatine-insensitive 1*), that may also significantly affect the expression of secondary metabolites (Xie et al., 1998, Schaller et al., 2000, Stintzi and Browse, 2000, Park et al., 2002). These pathways being potentially crucial for the production of glucosinolates also (Thines, Parlan and Fulton, 2019).

Moreover, as well as plant secondary metabolites being produced rhythmically, such as glucosinolate (Goodspeed et al., 2013b), rhythmic detoxification pathways may also be present in herbivorous animals, where lepidopteran midgut studies have shown the maintenance of peripheral circadian clocks, important in in the regulation of detoxification genes (Sauman and Reppert, 1998, Nobata et al., 2012, Zhang et al., 2021). In addition to lepidopteran and *P. xylostella* rhythmic behaviours being found (Minis and Pittendrigh, 1968, Pivnick et al., 1990, Sakamoto and Shimizu, 1994), these findings demonstrate how possible interactions between both the pest and host plant circadian clocks may inform herbivory resistance, as shown with the generalist pest *Trichoplusia ni* (Cabbage looper) (Goodspeed et al., 2012). It is however unclear how a specialist brassica feeding herbivore such as *P. xylostella* would react to these conditions and how its circadian rhythms may interact with host plant rhythmic cycles of secondary metabolites. Uncovering the circadian rhythms of pest species like *P. xylostella*, especially in feeding behaviour, will

likely aid with understanding treatment susceptibility and synergistic effects with natural plant defences due to the rhythmic cycles associated with detoxification genes and produced enzymes pests use during herbivory and for pesticide resistance (Bagheri et al., 2016, Zhang et al., 2021).

In this study, light/dark (L/D) and subsequent constant light (L/L) conditions are used to demonstrate the feeding rhythms of *P. xylostella* larvae on both *B. rapa* and *A. thaliana*. The differences between *P. xylostella* feeding on in-phase (IP) and out-of-phase (OP) *B. rapa* were investigated along with molecular genetic analysis to compare the expression profiles of both clock genes and a number of GSS genes in response to this feeding. Analysis of clock gene expression may show evidence for the presence of an active circadian clock in *P. xylostella* larvae while the profile of GSSs, which are likely to be the most influential on *P. xylostella*'s ability to disarm the MOB, may demonstrate any correlations between feeding behaviour, detoxification and both pest and host clocks. These genes' expression along with phenotypic behaviour in differing environmental conditions may show how *P. xylostella* cycles its defence against glucosinolate and anticipates such, including the time periods with the highest intensity of feeding and activity. Once these biological rhythms are known it may provide data to compliment future research into ways of modulating and exploiting *P. xylostella* circadian rhythms and herbivory damage through the enhancement of IPM strategies and or natural plant defences. *P. xylostella* fed on *A. thaliana* was also used to compare against *B. rapa*-fed larvae to show any differences in feeding rhythms between Brassicaceae species. In addition, both clock (*cca1-ox* (*circadian clock associated 1 - overexpressor*), *cca1-11/lhy-21/toc1-21*) and defence (*coi1-16, aos, opr3-1*) mutant *A. thaliana* were then used to uncover interactions between *P. xylostella* feeding and host plant clock and defence pathway functioning, potentially highlighting key effects of affected pathways and how

they might be exploited for altering pest-host interactions to control pest populations and reduce damage on plants.

4.3 Results

4.3.1 *P. xylostella* larval feeding on *B. rapa*

4.3.1.1 *B. rapa* leaf area loss during *P. xylostella* larval feeding

After recording *P. xylostella* larval feeding on IP *B. rapa* leaves for 24h in an L/D cycle, rhythmic changes in pixel area loss from *B. rapa* leaves was found. Shown in Fig. 4.1A, the highest level of leaf area loss in an h was recorded at ~2h after dark phase onset, showing peak activity around dark phase onset, including subjective (s) dark phase when referring to L/L conditions. The normalised rhythms of this leaf area loss over time is shown in the bottom graphs of Fig. 4.1, where the % of the total 24h feeding (leaf pixel area loss) in this condition was plotted against each h of feeding recorded. The rhythms found for IP L/D *P. xylostella* feeding leaf area loss and % of total herbivory showed dark phase to have significantly more feeding compared to light phase, as shown in Table 4.1.

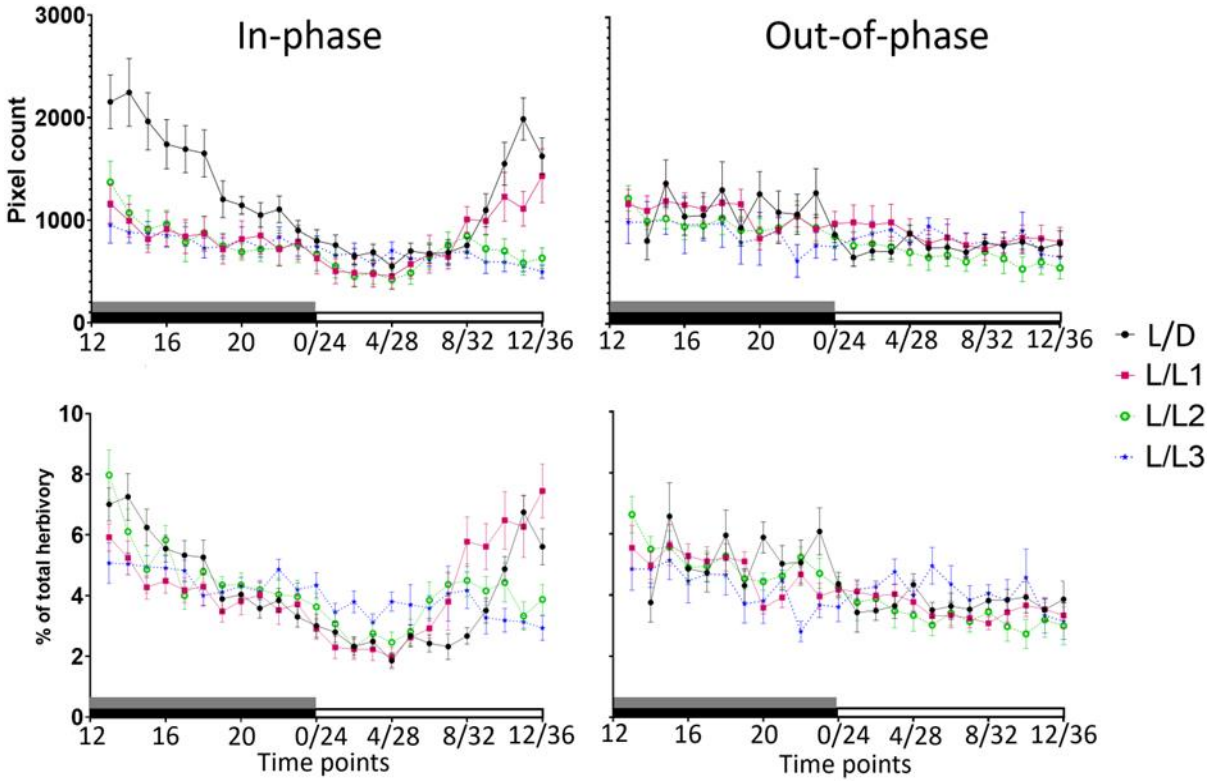


Figure 4.1 *P. xylostella* larval feeding rhythms on *B. rapa*.

Graphs show *P. xylostella* average feeding profiles. Larval *P. xylostella* exhibit rhythmic feeding patterns when feeding on both in and out-of-phase *B. rapa* leaves, however, with loss of rhythmicity over time in L/L and lacking anticipatory behaviour in out-of-phase conditions. Left, in-phase (IP) data; right, out-of-phase(OP) data. Line graphs with SEM error bars. Top showing pixel count loss of leaf tissue with bottom showing % of total herbivory that took place in each h denoted along the x-axis. Time points indicated as ZT/CT time points for L/D zeitgeber time (ZT) and L/L circadian time (CT). Black bar shows dark phase for L/D conditions and grey bar subjective (s) dark phase for L/L. Coloured lines represent L/D and deepening L/L conditions, with each number representing what progressive day in L/L was being recorded. L/L1 starts from first night loss in L/L conditions, L/L2 from second and so on.

The preference for significantly more feeding to take place during the (s) dark phase is also found from *P. xylostella* larvae feeding on OP *B. rapa* leaves, with leaf area loss and % of total herbivory rhythms shown in Fig. 4.1 also. OP L/D feeding and feeding in the second day of L/L (L/L2) showed significantly more leaf area loss in the (s) dark phase compared to (s) light phase as shown in Table 4.1. When normalizing leaf area loss against total

feeding to show overall rhythms in feeding, there was more significant differences found across IP feeding days. There was also a significantly larger % of total feeding taking place in the (s) dark phase. This was also found for OP % of total feeding, minus L/L3, shown in Table 4.1.

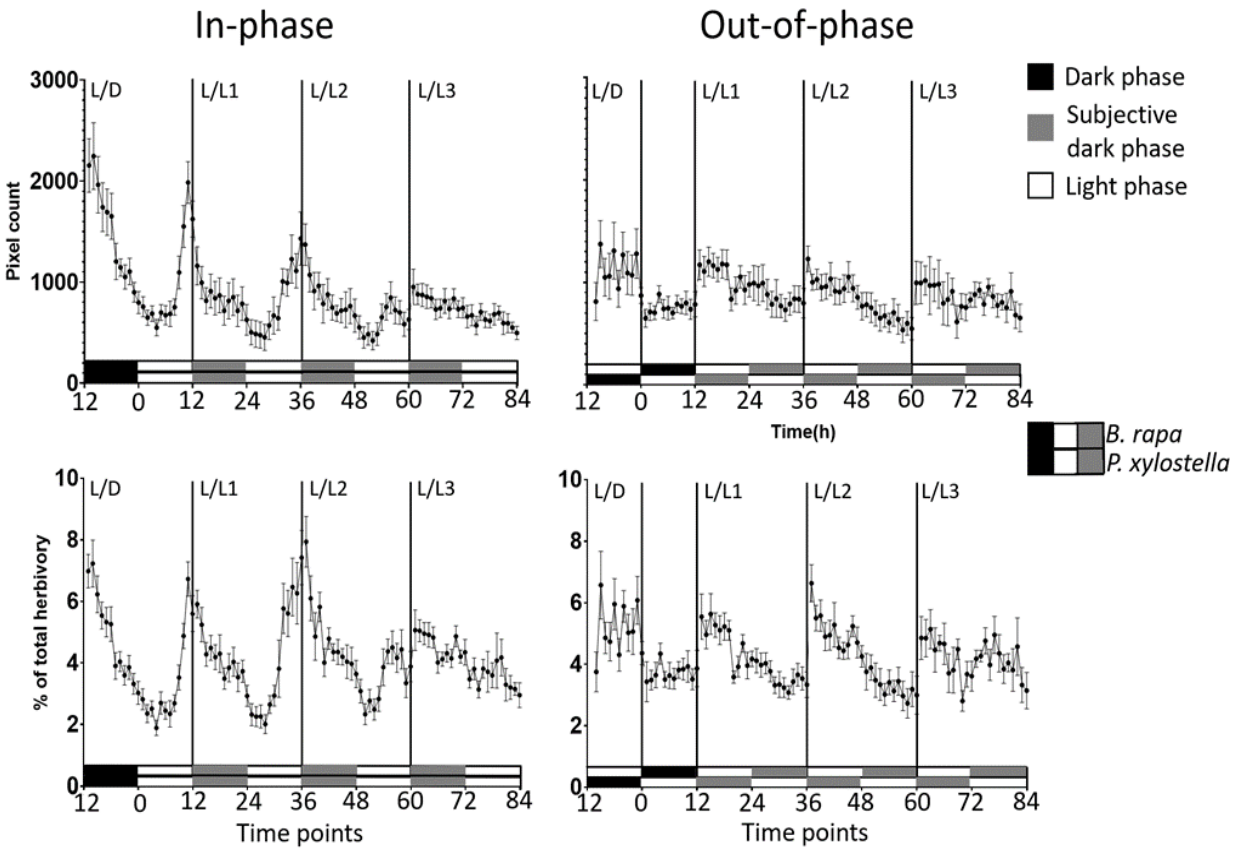


Figure 4.2 *P. xylostella* larval feeding rhythms over time on *B. rapa*.

Larval *P. xylostella* average feeding profiles plotted continuously, showing change over time and entrainment with *B. rapa* host plant material rather than difference between time points overlaid each other as in Fig. 4.1. Top graphs showing pixel count loss of leaf tissue with bottom showing % of total herbivory that took place in each h normalised to 24h periods. Time points indicated as ZT time points for L/D and CT time points for L/L. Black bar shows dark phase for L/D conditions and grey bar s dark phase for L/L. Black horizontal lines indicate separate recording periods. Top bar of the pair of time schedule bars shows the entrained L/D cycle of the host plant while bottom shows such for *P. xylostella* larvae. The first 24h period for OP is split due to plant material being recorded separately in dark conditions with *P. xylostella* larvae, then fresh 12h advanced OP plant material used in light conditions. This is to prevent any re-entrainment effects of plant material under L/D conditions.

The rhythmic profiles of IP and OP feeding is notably different, shown over time into extending L/L conditions in Fig. 4.2. IP feeding peaks around (s) dark phase onset, reaching a consistent trough between (s) light phase onset and middle (s) light phase (midday). Feeding also appears to increase during the second half of the light phase, most significantly in L/D and L/L1, showing significant linear regression slopes, but is still significantly higher compared to feeding in 1st half of light phase in L/L2, shown in Table 4.1. IP L/D and L/L1 anticipation slopes are also non-significantly different to each other. Feeding tends to decrease across (s) dark phase leading to feeding being notably higher around (s) dark phase onset than overall (s) dark phase. Total feeding 6h before and after (s) dark phase onset, demonstrating a period between (s) midday and (s) midnight, is shown in Table 4.1 and is significantly higher as a % of total herbivory than both s dark and s light phase in L/L1. When feeding on OP *B. rapa* leaves, *P. xylostella* larvae feeding shows high (s) dark phase feeding, being significantly higher in L/D and L/L2 conditions, including L/L1 and L/L3 for % of total herbivory, but lacks other aspects of the IP rhythmic profile. OP (s) dark phase feeding remains more consistently high compared to IP feeding profiles, more notably however, the increase in feeding towards the end of (s) light phase is lost. This leads to L/D and L/L2 light phase leaf area loss being significantly lower than (s) dark phase, differing from IP results. When comparing OP (s) midday-midnight, L/D and L/L2 is also significantly lower than % of total herbivory in (s) dark phase, with no 2nd half of light phase having a significantly higher % of total feeding. No OP *P. xylostella* larval feeding in any of the light conditions show significant slopes for increase in feeding towards (s) dark phase onset. However, IP L/L2 also lacks such, though having significantly more feeding in the 2nd half of s light phase compared to 1st.

Table 4.1 *P. xylostella* larval feeding on *B. rapa* data and analysis.

Data from Fig. 4.1, larval *P. xylostella* feeding under varying light phase conditions with ±SEM and significant difference groupings from statistical analysis. Pixel count loss from leaves is shown and the % of total pixel loss occurring within specific phases. The 12h periods matching subjective (s) dark phase, (s) light phase and (s) midday-midnight were compared. Midday-midnight includes the 6 time points before and after dark phase onset. * shows significant differences from dark phase equivalent, ^ shows significant differences from light phase equivalent. ✡ shows significant differences from the proportion of feeding in 1st ½ of light phase compared to 2nd and any significant linear regression slopes of such. # shows ANOVA showed significant difference over the whole group but no specific significant differences with Tukey's multiple comparisons test were found.

Condition (n)	Total	Sig	(s)Dark	Sig	(s)Light	Sig	(s)Midday -Midnight	Sig	(s)Dark %	Sig #	(s)Light %	Sig	(s)Midday-Midnight %	Sig	2nd ½ light %	Sig	Anticipation slope
IP L/D	30451	a	17888	a	11542	n/a	19541	a	58.44	a	41.424	n/a	63.192	a	63.36	abc	0.74☆
(16)	±2588		±1742		±1014*		±1931^		±2.340		±2.496*		±1.992^		±2.389☆		
OP L/D	20611	ab	13234	ab	9025	n/a	10530	b	61.74	a	44.544	n/a	49.716	b	49.94	ad	0.03
(8)	±2398		±1992		±698.0*		±1400		±3.540		±2.46*		±1.272*		±2.009		
IP L/L1	19450	ab	10102	b	9557	n/a	12004	b	49.896	a	49.956	n/a	63.816	ac	68.85	b	0.67☆
(12)	±3261		±1829		±1544		±1677		±3.564		±3.672		±3.204*^		±4.816☆		
OP L/L1	21903	ab	12843	ab	10232	n/a	11721	b	57.168	a	42.828	n/a	52.032	ab	47.20	d	0.04
(11)	±2389		±1193		±1492		±1030		±2.184		±2.184*		±1.752^		±2.315		
IP L/L2	17575	b	10292	b	7283	n/a	10214	b	58.092	a	41.916	n/a	58.236	ab	58.18	bd	-0.07
(12)	±2749		±1679		±1190		±1553		±2.856		±2.856*		±1.788^		±2.475☆		
OP L/L2	18553	ab	11817	ab	7966	n/a	9844	b	60.648	a	39.36	n/a	51.3	bc	45.72	d	-0.07
(12)	±2191		±1237		±1218*		±1081		±2.472		±2.472*		±3.156*^		±4.984		
IP L/L3	17623	b	9732	b	7391	n/a	9680	b	54.792	a	42.132	n/a	52.848	b	49.31	cd	-0.17
(18)	±1888		±1082		±697.0		±1306		±1.548		±2.556*		±2.448^		±3.581		
OP L/L3	19439	ab	10611	ab	9824	n/a	10516	b	50.76	a	49.248	n/a	51.396	ab	45.74	d	-0.15
(7)	±2339		±1995		±927.0		±1340		±3.252		±3.252		±1.152		±2.431		

Loss of IP rhythmic features can be seen across L/L conditions. In addition to using CircaCompare results, reduction in rhythmic amplitude can be tracked over time visually in Fig. 4.2. The change in proportion of feeding activity leading up to (s) dark phase onset can also be seen in Table 4.2, showing how cosinusoidal curve peaks shift towards and away from (s) dark phase onset with L/L1 being significantly earlier than L/D and L/L2. By L/L3 however the phase peak shifts significantly towards the middle of (s)dark phase, demonstrating a loss in late (s) light phase activity. L/L3 shows somewhat decreased feeding across the 24h day with a significantly lower rhythmic amplitude than other IP conditions. L/L2 rhythmic amplitude is both significantly higher than L/L3 and lower than L/D and L/L1. This demonstrates a gradual decline in rhythmicity as *P. xylostella* larvae feed in further L/L conditions. Yet, L/L3 was still found to have significant rhythmicity in IP conditions, however, this was lost in OP, with OP L/L3 being arrhythmic. Though rhythmicity is lost by L/L3, gradual loss in rhythmicity is less present in OP fed *P. xylostella*. Rhythmicity significance values do not trend downwards into L/L, like seen in IP, with rhythmic amplitude also showing no significant differences. This leads to rhythmic amplitude in OP L/D and L/L1 being significantly lower than IP counterparts while L/L2 is non-significantly different between the two conditions. These findings show a notably different relationship between rhythmic features of IP and OP with light conditions. Table 4.2 also shows no significant differences in phase peak between OP rhythms with each being significantly later than respective IP results, with cosinusoidal curve peak being found at the middle of (s) dark phase (midnight), leading to (s) midday-midnight in OP not being significantly higher than (s) dark phase feeding.

Table 4.2 CircaCompare rhythmic features of *P. xylostella* larval feeding on *B. rapa*.

Table showing CircaCompare % of total herbivory results and comparisons between both IP and OP conditions and light conditions. Significant differences in % of total herbivory rhythmic amplitude, mesor and phase peak between light conditions is shown through lettering system and * for showing differences between IP and OP

counterparts. Rhythm *P* equates to the *P* value of data's fit to cosinudsoidal curve, Amplitude is a measure of amount of change between peak and trough of calculated curve, Mesor equates to a rhythm-adjusted mean, Phase peak is shown in ZT time points for L/D and CT for L/L, Mesor should be normalized with no significant differences.

Condition In-phase	Rhythm *P*	Amplitude	Sig	Condition Out-of-phase	Rhythm *P*	Amplitude	Sig
L/D	2.20^{-42}	2.09	a	L/D	1.97^{-8}	0.91*	a
L/L1	4.00^{-21}	1.77	a	L/L1	1.51^{-11}	0.85*	a
L/L2	3.88^{-14}	1.25	b	L/L2	4.15^{-13}	1.10	a
L/L3	1.32^{-6}	0.59	c	L/L3	Arrhythmic	n/a	

Condition In-phase	Mesor	Sig	Phase peak	Sig	Condition Out-of-phase	Mesor	Sig	Phase peak	Sig
L/D	4.12	a	14.62	a	L/D	4.38	a	18.63*	a
L/L1	4.16	a	12.60	b	L/L1	4.17	a	17.48*	a
L/L2	4.17	a	14.63	a	L/L2	4.17	a	18.00*	a
L/L3	4.10	a	16.97	c	L/L3	n/a		n/a	

Table 4.1 indicates that total daily feeding changes across the conditions examined. As can be seen from Fig. 4.1, IP L/D has the highest amount of leaf area loss from *P. xylostella* feeding with a significant increase relative to IP L/L2 and L/L3, indicating that total feeding decreases in extended L/L conditions. However, none of the conditions in OP showed significant differences in total daily feeding, showing that loss of the dark phase had a reduced impact on feeding totals in OP conditions.

4.3.1.2 *B. rapa* fed *P. xylostella* larvae relative expression of clock and GSS genes

The rhythmic profile of clock genes *per* and *tim* relative expression against *Ef1α* (*Elongation factor 1 α*), shown in Fig. 4.3A, closely match each other with CircaCompare cosinusoidal curve fitting showing no significant difference between rhythmic features in both IP and OP conditions, shown in Table 4.3. However, there appears to be minor changes to the rhythmic profile in L/L circadian time (CT) points compared to zeitgeber

time (ZT) points, including with the relative expression of GSS genes, shown in Fig. 4.3B, with the combined primer *Sulf2+3* results for IP feeding shown in Suppl. Fig. 1 and Suppl. Table 1.

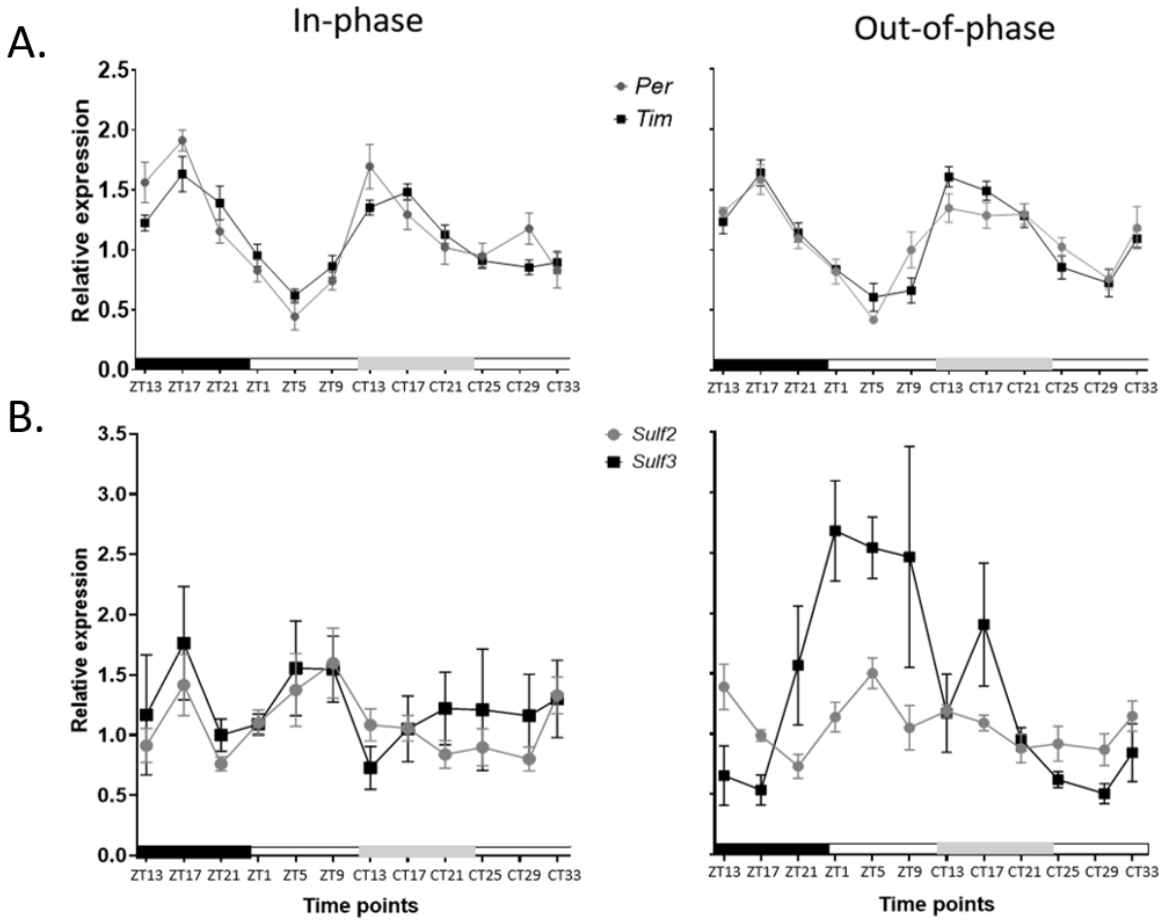


Figure 4.3 In-phase and out-of-phase qPCR relative expression of larval *P. xylostella* clock and sulfatase genes.

Larval *P. xylostella* rhythmic mRNA expression levels of *per* and *tim* maintain rhythmicity in L/L in both IP and OP feeding conditions. Rhythmic expression of GSS genes is less clear however. A. Line graph with SEM showing relative expression of clock genes *per* and *tim* over time relative to the *Ef1α* housekeeping gene, B, showing such for GSS genes, with 12/12 L/D zeitgeber time denoted by black bar for dark phase and ZT time points, and a light grey bar for circadian dark phase and CT time points in L/L.

Table 4.3 In-phase larval *P. xylostella* qPCR relative expression of clock and sulfatase genes.

CircaCompare results from 48h rhythms of clock and GSS gene relative expression levels shown along with ANOVA and Kruskal-Wallis tests of 24h L/D and L/L collated rhythms and comparisons between L/D and L/L day time points. 2-way ANOVA results also shown for combination between effects of time over 24h day and L/D or L/L conditions, in addition to whether there is any significant interaction between the two variables accounting for any variation in relative expression. 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 ZT time points and respective CT time points where the peak of fitted curve occurs.

In-phase

Transcript	Rhythmicity	Mesor	Amplitude	Phase peak	ANOVA 24h
per	2.29^{-3}	1.13	0.47	15.80	*P* $<1x10^{-4}$
tim	5.40^{-6}	1.11	0.40	17.01	*P* $<1x10^{-4}$
Sulf2	Arrhythmic				*P <0.05*
Sulf3	Arrhythmic				*P >0.05*

ANOVA	*Per* L/D	*Per* L/L	*Tim* L/D	*Tim* L/L	*Sulf2* L/D	*Sulf2* L/L	*Sulf3* L/D	*Sulf3* L/L
P value	*P* <0.0001	*P <0.01*	*P* <0.0001	*P* <0.0001	*P >0.05*	*P >0.05*	*P >0.05*	*P >0.05*
Time points	Sig	Sig	Sig	Sig	Sig	Sig	Sig	Sig
ZT13/CT13	ab	a	ab	ab				
ZT17/CT17	a	ab	a	a				
ZT21/CT21	bc	b	ab	bc				
ZT1/CT25	cd	b	ab	c				
ZT5/CT29	d	ab	c	c				
ZT9/CT33	cd	b	bc	c				

2-way ANOVA	*Per* L/D vs L/L %	*P* value	*Tim* L/D vs L/L %	*P* value	*Sulf2* L/D vs L/L %	*P* value	*Sulf3* L/D vs L/L %	*P* value
Condition	0.4775	*P >0.05*	0.01941	*P >0.05*	4.096	*P* <0.05	n/a	*P >0.05*
Time	53.14	*P* <0.0001	63.19	*P* <0.0001	19.35	*P* <0.05	n/a	*P >0.05*
Interaction	17.53	*P* <0.001	5.457	*P* >0.05	7.094	*P* >0.05	n/a	*P >0.05*

Despite these differences, the 24h compiled analysis of average data points showed significant change over time as indicated in Table 4.3 and rhythmicity for 48h rhythms of relative expression of IP *per* and *tim* and OP *per*, *tim* and *Sulf2* in Table 4.4. Table 4.3 and 4.4 also shows change in relative expression between data within L/D and L/L conditions individually. IP *per* expression peaks at ~ZT17, being significantly higher than all other time points after such, with ZT5 being the trough, though is none significantly different from neighbouring light phase time points. In Suppl. Fig. 4, CircaCompare analysis plots the phase peak for IP L/D *per* relative expression as ZT16.48 (48h double curve plot shows 15.80). When moving into L/L conditions and CT time points however the rhythmic profile changes, with the change over time (Amplitude) being lower significantly lower than in L/D conditions. Phase peak and mesor remains non-significantly different however. The significant differences between specific time points are also reduced with significant differences only found between CT13 against CT21, CT25 and CT33. This demonstrates that *per* rhythmicity is reduced in L/L conditions, also indicated by lower rhythmicity significance, as shown in Suppl. Fig. 4. The majority of the variation seen in IP *per* results is attributed to change in time points according to 2-way ANOVA results shown in Table 4.3, however, there is a significant interaction between time and condition. The relative expression of *tim*, though showing a similar rhythmic profile, has different effects of change between L/D and L/L conditions, with the two-way ANOVA attributing significant variation to changes in time points. The peak time point is still shown as ZT17, however is only significantly higher than ZT5 and 9, yet, though having a significant drop in rhythmic amplitude in L/L, the drop is less severe as seen in Suppl. Fig. 4. This is also evident when comparing *tim* L/L time points, where the CT17 peak remains significantly higher than all following time points. This is similar to *per* L/D results, with CircaCompare phase peak found to be the same, around ZT17.60 for L/D and CT16.29 for L/L (17.01 for 48h double curve plot).

Table 4.4 Out-of-phase larval *P. xylostella* qPCR relative expression of clock and sulfatase genes.

CircaCompare results from 48h rhythms of clock and GSS gene relative expression levels shown along with ANOVA and Kruskal-Wallis tests of 24h L/D and L/L collated rhythms and comparisons between L/D and L/L day time points. 2-way ANOVA results also shown for combination between effects of time over 24h day and L/D or L/L conditions, in addition to whether there is any significant interaction between the two variables accounting for any variation in relative expression.

Out-of-phase

Transcript	Rhythm *P*	Mesor	Amplitude	Phase peak	ANOVA 24h
per	1.37^{-11}	1.09	0.38	16.12	*P* <$1x10^{-4}$
tim	9.17^{-17}	1.10	0.46	16.41	*P* <$1x10^{-4}$
Sulf2	7.99^{-3}	1.07	0.16	9.75	*P* <0.05
Sulf3	Arrhythmic				*P* >0.05

ANOVA	*per* L/D	*per* L/L	*tim* L/D	*tim* L/L	*Sulf2* L/D	*Sulf2* L/L	*Sulf3* L/D	*Sulf3* L/L
P value	*P* <0.0001	*P* <0.01	*P* <0.001	*P* <0.0001	*P* <0.01	*P* >0.05	*P* <0.01	*P* <0.05
Time points	Sig	Sig	Sig	Sig	Sig	Sig	Sig	Sig
ZT13/CT13	ab	a	ab	a	a		a	ab
ZT17/CT17	b	a	b	a	ab		a	a
ZT21/CT21	ac	a	ab	a	b		ab	ab
ZT1/CT25	cde	ab	a	b	ab		b	ab
ZT5/CT29	d	b	a	b	a		b	b
ZT9/CT33	ae	ab	a	b	ab		ab	ab

2-way ANOVA	*per* L/D vs L/L %	*P* value	*tim* L/D vs L/L %	*P* value	*Sulf2* L/D vs L/L %	*P* value	*Sulf3* L/D vs L/L %	*P* value
Condition	2.097	*P* <0.05	3.636	*P* <0.01	3.227	*P* <0.05	9.792	*P* <0.01
Time	53.04	*P* <0.0001	64.20	*P* <0.0001	21.00	*P* <0.05	5.091	*P* >0.05
Interaction	7.059	*P* >0.05	5.999	*P* <0.05	17.41	*P* <0.05	30.52	*P* <0.0001

OP *per* and *tim* results shown in Table 4.4 are similar with *per* and *tim* OP relative expression levels over 48h, using cosinusoidal double curve fitting, showing peak phase at ~ZT/CT16 (compared to 15.8 for IP *per* and 17.01 for IP *tim*). Other rhythmic features are

also non-significantly different, apart from *per* L/D amplitude, shown in Table 4.5. OP *per* L/D peak time point, as shown in Suppl. Fig. 5, is similar to L/D *per* at ZT17 which is significantly higher than following time points, with the trough being ZT5. A clear peak and trough is lost in L/L conditions with CT29 being significantly lower than s dark phase time points, maintaining a trough but without a clear peak point maintained. CircaCompare analysis in Suppl. Fig. 5 shows that the OP *per* peak is non-significantly different however at ZT16.11 and CT16.09 respectively. However, the CircaCompare cosinusoidal curve fitting analysis does show a significant drop in rhythmic amplitude similar to the results seen with IP *per*. 2-way ANOVA shows that though time again contributes a significant amount of the variation recorded, in OP the change in L/D and L/L conditions is responsible for significant amounts of the variation. OP *tim* shows a similar set of results and comparisons, shown in Table 4.4. However, when looking at the specific differences between time points, a clear peak time at ZT17 is lost in L/L and in both L/D and L/L there is no clear trough. Through CircaCompare analysis, rather than a drop in amplitude between L/D and L/L, OP *tim* relative expression was shown to have a significantly increased mesor and earlier phase peak in L/L, at CT15.57 compared to ZT17.19 in L/D conditions. In addition to significant variation attributed to changes in time points, two-way ANOVA analysis found significant variation from both changes in condition and interaction between both condition and time, differing from IP *tim* results. When comparing IP and OP *per* and *tim* results directly, as shown in Table 4.5, there was only significant differences found between the cosinusoidal curve fitting of *per* L/D data, with IP relative expression rhythms having significantly higher rhythmic amplitude, also shown in Suppl. Fig. 6, demonstrating similar rhythmic profiles.

Table 4.5 CircaCompare comparison between in and out-of-phase relative expression.

CircaCompare results of comparison between respective gene and light cycle conditions between IP and OP fed *P. xylostella* larvae. * showing significant differences between rhythmic features, # to highlight that *Sulf2* in-phase expression under L/D conditions didn't show significant rhythmicity.

In-phase vs Out-of-phase

per L/D			*tim* L/D			*Sulf2* L/D#		
Rhythmic feature	In-phase vs Out-of-phase	*P* value	Rhythmic feature	In-phase vs Out-of-phase	*P* value	Rhythmic feature	In-phase vs Out-of-phase	*P* value
Rhythm *P*	1.37^{-10} vs 7.27^{-10}	n/a	Rhythm *P*	2.03^{-9} vs 9.84^{-10}	n/a	Rhythm *P*	0.070 vs 0.011	n/a
Mesor	1.11 vs 1.04	0.286	Mesor	1.11 vs 1.18	0.111	Mesor	1.19 vs 1.13	0.612
Amplitude*	0.69 vs 0.50	0.041	Amplitude	0.48 vs 0.44	0.861	Amplitude	0.24 vs 0.25	0.991
Phase Peak	16.48 vs 16.11	0.532	Phase Peak	17.60 vs 15.57	0.526	Phase Peak	8.34 vs 7.80	0.834
per L/L			*tim* L/L			*Sulf2* L/L		
Rhythmic feature	In-phase vs Out-of-phase	*P* value	Rhythmic feature	In-phase vs Out-of-phase	*P* value	Rhythmic feature	In-phase vs Out-of-phase	*P* value
Rhythm *P*	0.015 vs 5.30^{-4}	n/a	Rhythm *P*	5.03^{-9} vs 9.84^{-10}	n/a	Rhythm *P*	0.014 vs 0.010	n/a
Mesor	1.16 vs 1.15	0.882	Mesor	1.10 vs 1.18	0.137	Mesor	1.00 vs 1.02	0.839
Amplitude	0.25 vs 0.26	0.985	Amplitude	0.33 vs 0.44	0.093	Amplitude	0.20 vs 0.17	0.799
Phase Peak	14.37 vs 16.09	0.322	Phase Peak	16.29 vs 15.57	0.301	Phase Peak	23.67 vs 12.71	0.625

Comparisons between GSS genes, shown in Fig. 4.3B, show how the relative expression levels of *Sulf2* and *Sulf3* change over time compared against *Ef1α* reference gene. IP *Sulf2+3* combined primer results are shown in Suppl. Fig. 1 and Suppl. Table 1, showing a similar profile to other GSS specific genes, but lacking any significant differences between time points, therefore, as *Sulf2* and *Sulf3* primers were successfully used in qPCR (Quantitative polymerase chain reaction), analysis was carried out on the specific primer results only. *Sulf2* and *Sulf3* IP L/D results at first show bimodal peaks at ZT17 and ZT9, nevertheless, statistical testing shows no significant differences between any two points,

including in L/L conditions, shown in Table 4.4. Averaged 24h *Sulf2* rhythms showed significant change over time, however, neither individual IP L/D nor L/L days showed significant changes. CircaCompare analysis showed the 48h rhythm as arrhythmic, including the L/D day. L/L IP *Sulf2* expression was found to be rhythmic however, with 2-way ANOVA results showing changes in time and condition contributed a significant proportion of the variation. IP *Sulf3* results showed similarly arrhythmic results, lacking evidence of rhythms in expression, with no significant change over time found for 24h averaged rhythms and no significance calculated by 2-way ANOVA. CircaCompare analysis failed to detect any significant rhythms in either 48h or individual IP Sulf3 L/D or L/L rhythms also.

In contrast, OP *Sulf2* and *Sulf3* showed more significant differences and rhythms. *Sulf2* showed significant change over time with 24h collated rhythms. In addition compared to IP, CircaCompare showed significant rhythmicity over the 48h *Sulf2* time course, though with a notably lower rhythmicity and amplitude compared to 48h clock gene time courses. Also shown in Table 4.4, OP *Sulf2* showed significant change over time in L/D conditions with ZT13 and ZT5 peaks in relative expression significantly higher than ZT21 as a trough. CircaCompare analysis showed both L/D and L/L time courses had significant rhythmicity however with a significantly different phase peak between the two. L/L *Sulf2* phase peak is shifted ~5h near to (s) dark phase onset (CT12.71) compared to L/D peak that occurs ~4h before such (ZT7.80). Significant variation was found to occur due to changes in both time and condition along with a significant interaction effect between the two using 2-way ANOVA. OP *Sulf3* was shown to have the highest contribution of variation from an interaction effect out of all conditions, additionally showing no significant effect from change in time, as shown in Table 4.4. OP *Sulf3* 48h relative expression rhythms were found to be arrhythmic by CircaCompare analysis along with collated 24h data showing no significant change over time. Individual testing on OP L/D

and L/L relative expression however produced significant results, dissimilar to IP *Sulf3*. Shown in Suppl. Fig. 5, there was widely differing changes in expression over time between L/D and L/L. *Sulf3* under OP L/D conditions peaked around the middle of light phase, however peaked at CT17 in L/L in the opposite 12h phase. Further to this, CircaCompare analysis also shows significant difference in both phase peak and mesor, with the phase peak being ~12h phase shifted.

Comparisons between IP and OP *Sulf2* rhythms, shown in Table 4.5 and Suppl. Fig. 6, show no significant differences, though IP *Sulf2* L/D lacks significant rhythmicity either way. However, no significant differences were found comparing *Sulf2* time points with each other in Table 4.6. *Sulf3* on the other hand, though could not be compared directly due to lack of rhythmicity in IP *Sulf3*, showed ZT17 and ZT1 time points to be significantly different between IP and OP conditions, specifically where IP *Sulf3* expression peaked at ZT17 and ZT1 at the beginning of the light phase in OP. Suppl. Fig. 7 shows comparison between the relative expression rhythms of OP *Sulf2* and Sulf3, finding OP L/D *Sulf3* to have significantly different rhythmic amplitude and mesor, phase peak is non-significantly shifted however, with the only significant difference between the two GSS genes in L/L being rhythmic amplitude.

Table 4.6 Comparison between relative expression levels of IP and OP *Sulf2* and *Sulf3* time points.

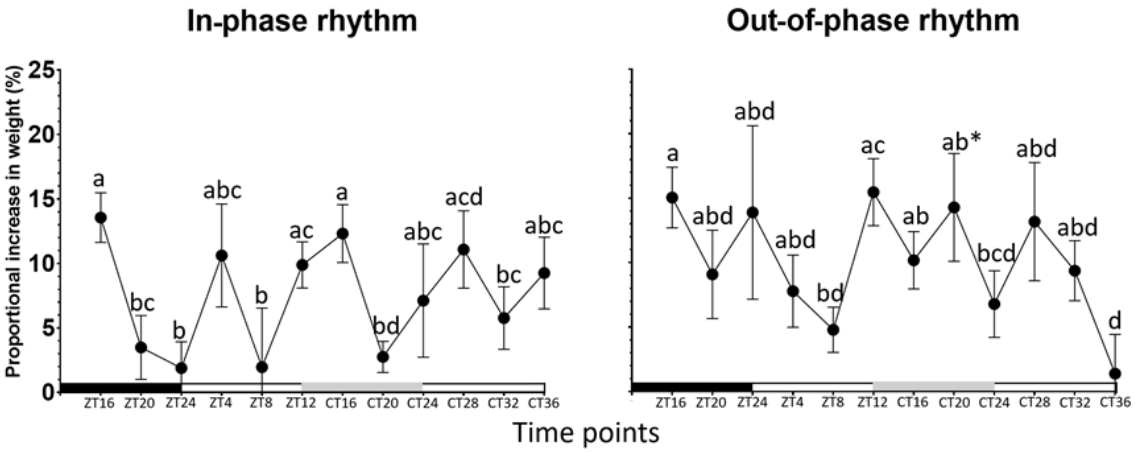
Sulf2 and *Sulf3* time points compared to their respective IP and OP equivalent. Average relative expression shown with the *P* value of t-test used to compare the two sets of data of each individual time point. Mann-Whitney U tests were used for *Sulf2* ZT5 and *Sulf3* ZT13, ZT9, CT17, CT25 and CT33. * highlights significant differences between IP and OP data.

Sulf2 Time point	IP vs OP	*P* value	Sulf3 Time point	IP vs OP	*P* value
ZT13	0.914 vs 1.390	0.0674	ZT13	1.168 vs 0.656	0.3939
ZT17	1.416 vs 0.988	0.1649	ZT17*	1.762 vs 0.537	0.0305
ZT21	0.762 vs 0.733	0.8016	ZT21	1.001 vs 1.568	0.2923
ZT1	1.101 vs 1.140	0.8147	ZT1*	1.091 vs 2.682	0.0077
ZT5	1.375 vs 1.502	0.1775	ZT5	1.555 vs 2.542	0.0609
ZT9	1.598 vs 1.052	0.1660	ZT9	1.547 vs 2.466	0.3586
CT13	1.084 vs 1.191	0.5094	CT13	0.729 vs 1.173	0.2564
CT17	1.058 vs 1.082	0.8034	CT17	1.053 vs 1.907	0.0931
CT21	0.840 vs 0.881	0.8131	CT21	1.221 vs 0.957	0.4211
CT25	0.899 vs 0.920	0.9244	CT25	1.211 vs 0.622	0.5887
CT29	0.802 vs 0.871	0.6819	CT29	1.161 vs 0.508	0.0943
CT33	1.330 vs 1.148	0.3950	CT33	1.300 vs 0.847	0.4848

4.3.1.3 *B. rapa*-fed *P. xylostella* larval weight gain

Both IP and OP *B. rapa* feeding showed significant change over time found in *P. xylostella* larval weight gain, shown in Fig. 4.4A. CircaCompare analysis showed no significant rhythmicity in either sets of data, however, notable shifts in higher and lower levels of weight increase were found.

A.



B.

Condition	IP Dark LD	IP Light LD	IP Dark LL	IP Light LL	OP Dark LD	OP Light LD	OP Dark LL	OP Light LL
AVG weight gain/h (%)	2.064 ±0.343	1.903 ±0.258	2.066 ±0.391	2.252 ±0.264	3.218 ±0.430	2.017 ±0.470	2.746 ±0.571	2.487 ±0.648

Condition	In LD	In LL	Out LD	Out LL	In	Out
AVG weight gain/h (%)	1.984 ±0.279	2.159 ±0.207	2.617 ±0.406	2.617 ±0.601	2.071 ±0.135	2.617 ±0.489

Figure 4.4 Larval *P. xylostella* proportional weight gain over time.

Larval weight gain shows somewhat similar trends to larval feeding assays but are more variable and do not show significant rhythmicity.

A. Line graphs with SEM error bars (n = 8) showing larval *P. xylostella* weight gain over time in L/D and L/L conditions, with larvae fed in-phase *B. rapa* shown on the left and those fed out-of-phase *B. rapa* on the right. Lettering system to show t-test significant differences between average proportional increases in weight at each time point. IP ZT4 and OP ZT16 and ZT24 were not normally distributed so was compared to other time points using Mann-Whitney U tests. 12/12 L/D zeitgeber time denoted by ZT time points and a light grey bar for circadian dark phase and CT time points in L/L. Data calculated by subtracting the previous recorded proportional amount of weight gain, recorded in sets of 12h. * shows significant difference between OP time point average and respective IP time point.

B. Table showing average proportional weight gain divided by h in conditions with SEM error. IP refers to in-phase and OP refers to out-of-phase. Bottom part of table shows pooled averages.

IP weight gain was significantly higher at the beginning of dark phase compared to later dark phase time points, including between s dark phase L/L CT16 and CT20 time points,

with both ZT16 and CT16 non-significantly different. The final weight gain time point for light phase (ZT12/CT36) in IP L/D and L/L conditions rises and is non-significantly different from ZT16, showing somewhat similar results to *B. rapa* rhythmic feeding profiles. However, in both L/D and L/L conditions there appears to be an increase in weight gain at ZT4 and around the L/L equivalent time point CT28. However, variation is high across the time courses, therefore these time points are non-significantly different from their surrounding time points. OP L/D weight gain has a similar downwards trend from ZT16 weight gain, however, has increased variation so only ZT16 is significantly different from ZT8, including CT24 and CT36 in L/L conditions, before rising again at ZT12. After this rise, weight gain stays high for the rest of the OP time course until dropping at CT36, being significantly lower than ZT16, ZT12 and CT20. When comparing time points between IP and OP time courses, CT20 is also the only time point significantly different between IP and OP weight gain, dropping in the former while staying high in the latter. Fig. 4.4B shows comparisons between weight gain during different phases and in specific conditions. Though all highest levels of average and total weight gain occur in OP conditions, due to large levels of variation no significant differences were found between groupings of data.

4.3.2 *P. xylostella* larval feeding on *A. thaliana*

4.3.2.1 Col-0 and mutant *A. thaliana* feeding

Results from Col-0 wild type (WT) *A. thaliana* leaf area loss showed significant rhythmic changes over time with certain similarities to *B. rapa* rhythmic feeding profiles. In both L/D and L/L, shown in Fig. 4.5A, leaf area loss starts high after (s) dark phase onset then trends downwards towards the first half of (s) light phase where the trough occurs. Also seen in % of total herbivory over time where it is evident there is reduced change compared to *B. rapa* feeding. CircaCompare analysis, shown in Suppl. Fig. 8 and Suppl.

Table 2, shows significantly increased rhythmic amplitude in *P. xylostella* feeding on *A. thaliana* leaves in L/L conditions compared to L/D. Though there is increase in rhythmic amplitude, no increase in % of total feeding occurs around the second ½ of light phase, which would otherwise shift the phase peak further towards (s) dark phase onset like seen in *B. rapa* feeding in L/D and L/L1 conditions. Moreover, the phase peak between *A. thaliana* L/D and L/L is significantly delayed, with L/L peak feeding occurring further towards the middle of s dark phase (midnight). However, the slope of proportion of total feeding increasing towards (s) dark phase onset is significant in both L/D and L/L WT *A. thaliana* feeding, additionally, though L/L shows a delayed phase peak, the anticipation slopes are not significantly different. When comparing *A. thaliana* L/D and L/L feeding rhythms directly to *B. rapa*, L/D shows significantly reduced rhythmic amplitude when larval *P. xylostella* are feeding on *A. thaliana* leaves while other rhythmic features remain unchanged. In L/L feeding however, the amplitude remains non-significantly different, but phase peak is significantly delayed, ~5h from s dark phase onset (CT16.98) compared to *B. rapa* peak at ~0.5h (CT12.60).

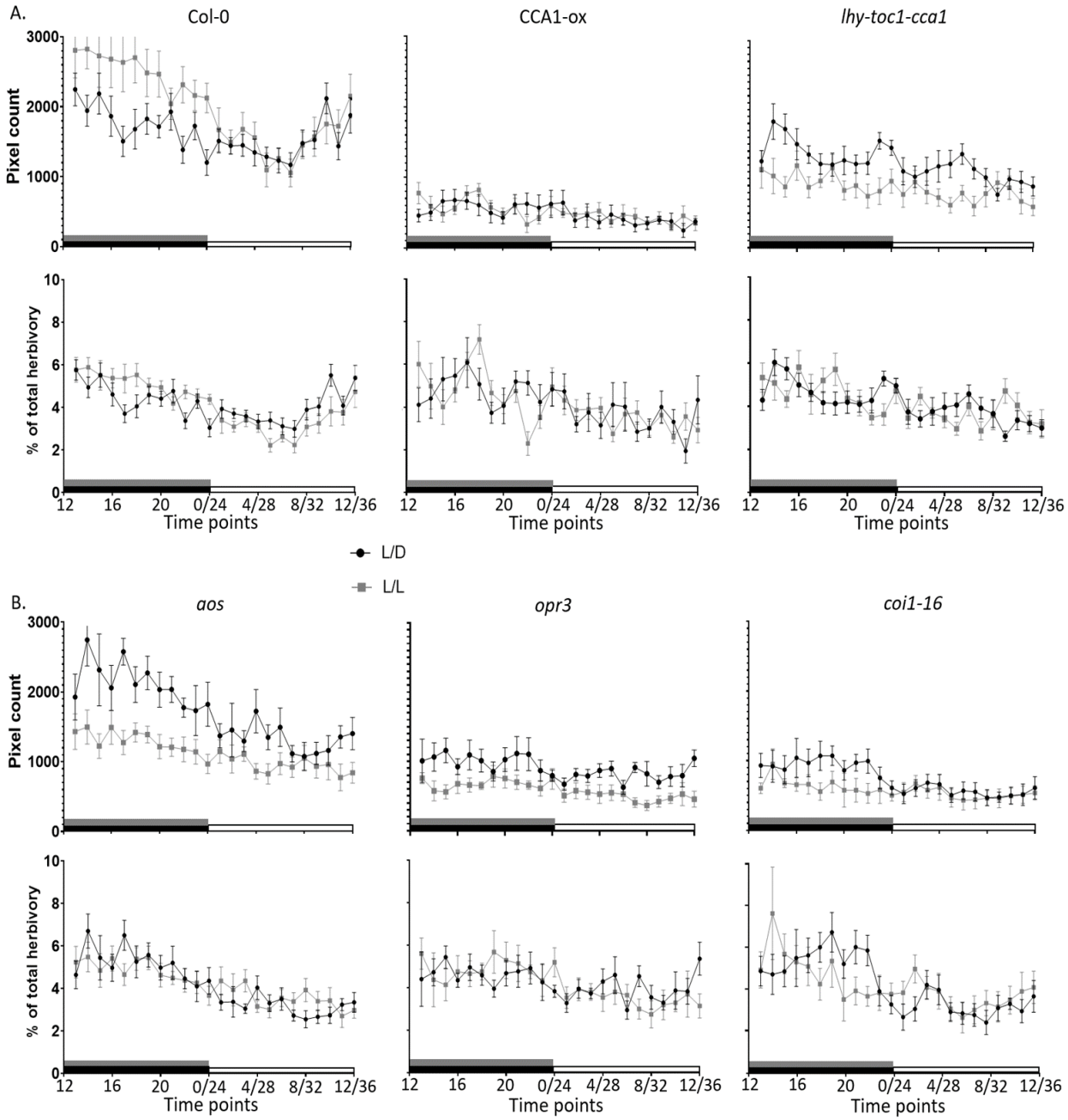


Figure 4.5 Larval *P. xylostella* feeding on Col-0 and *cca1-ox A. thaliana.*

Larval *P. xylostella* exhibit rhythmic feeding patterns when feeding on Col-0 *A. thaliana* similar to those found on *B. rapa* leaves, however, feeding rhythms are altered on mutant *A. thaliana* leaves.

A. Larval *P. xylostella* average feeding profiles on *A. thaliana* WT and clock mutants, B. Larval *P. xylostella* average feeding profiles on *A. thaliana* JA mutants. Line graphs with SEM error bars. Top showing pixel count loss of leaf tissue with bottom showing % of total herbivory that took place in each h. Time points indicated as ZT/CT time points for L/D zeitgeber time (ZT) and L/L circadian time (CT).

Black bar shows dark phase for L/D conditions and grey bar subjective dark phase for L/L. Black lines indicated L/D conditions and grey lines indicate L/L.

Table 4.7 CircaCompare comparison between L/D and L/L larval *P. xylostella* feeding rhythms on WT and mutant *A. thaliana*.

Table showing CircaCompare % of total herbivory results and comparisons between both L/D and L/L conditions and *A. thaliana* genotypes. WT referring to Col-0 wild-type *A. thaliana* and TM to *cca1-11/lhy-21/toc1-21* triple mutant. Significant differences in % of total herbivory rhythmic amplitude and phase peak between light conditions is shown through *. Rhythm *P* equates to the *P* value of data's fit to cosinudsoidal curve, Amplitude is a measure of amount of change between peak and trough of calculated curve, Phase peak is shown in ZT time points for L/D and CT for L/L, Mesor should be normalized with no significant differences.

Condition L/D	Rhythm *P*	Amplitude	Phase peak	Condition L/L	Rhythm *P*	Amplitude	Phase peak
WT	8.97^{-10}	0.83	14.61	WT	4.64^{-22}	1.47*	16.98*
CCA1-ox	8.53^{-5}	0.91	18.94	CCA1-ox	4.13^{-6}	0.93	17.68
TM	3.04^{-4}	0.56	18.73	TM	1.35^{-5}	0.76	17.04
aos	2.29^{-14}	1.43	17.99	*aos*	3.48^{-8}	0.92*	18.21
opr3	4.06^{-3}	0.53	16.87	*opr3*	1.82^{-6}	0.98	19.27
coi1-16	1.40^{-10}	1.32	18.27	*coi1-16*	2.27^{-4}	0.96	16.39

When comparing WT *A. thaliana* feeding profiles to clock mutant feeding, shown in Fig. 4.5A and Table 4.7 and 4.8, there are significant differences, most notably the reduction in pixel area loss from *A. thaliana* leaves with *cca1-ox* mutant leaves, having significantly reduced total feeding (leaf area loss) shown in Table 4.8. *cca1-11/lhy-21/toc1-21* (*circadian clock associated 1-11/late elongated hypocotyl-21/timing of CAB expression 1-21*) triple mutant (TM) leaf area loss was also reduced, however with only L/D and L/L feeding significantly lower than WT L/L feeding and TM L/D feeding being non-significantly different from WT L/D. Certain aspects of the rhythmic profile are changed,

with Suppl. Table 2 showing CircaCompare analysis results from comparing L/D and L/L conditions individually between WT and clock mutants. In L/D and L/L conditions clock mutants lose increased feeding towards the end of (s) light phase, also seen in Fig. 4.5A, leading to significantly delayed phase peaks towards the middle of dark phase in L/D (6.94h and 6.73h from dark phase onset in *cca1-ox* and TM mutants respectively) and the loss of significant anticipation slopes in both conditions. Both clock mutant rhythmic amplitudes are not significantly different to WT in L/D, in contrast, L/L conditions show non-significantly different phase peaks but significantly reduced rhythmic amplitude individually between L/D and L/L. The loss of (s) dark phase onset anticipation and rhythmic amplitude is similar to results between IP and OP *B. rapa* feeding, where amplitude and phase peak was significantly altered and L/D and L/L1 significant anticipation slope was lost. In WT *A. thaliana* feeding there is significantly more feeding taking place in s dark phase in L/L conditions, with significantly more feeding also found between (s) midday-midnight in both L/D and L/L conditions compared to (s) light phase feeding alone, shown in Table 4.8. The same significant differences are found when comparing the data normalized as % of total herbivory. However, unlike in *B. rapa* feeding, there is not a significantly larger proportion of feeding taking place in the 2nd half of (s) light phase compared to the 1st, though when compared to WT *A. thaliana* L/D conditions this doesn't result in a shifted phase peak, unlike in L/L, shown in Suppl. Fig. 9. When feeding on *cca1-ox* mutant leaves, in addition to significantly reduced total feeding compared to WT, there is no significant differences in feeding between phases in L/D, though in L/L (s) light phase feeding is significantly lower than both s dark phase and s midday-midnight. This including again no significant differences in proportion of feeding across the 2nd ½s of s light phase, shown in Table 4.8. This is also seen for TM feeding, however more significant differences are found in L/D feeding in contrast to WT and *cca1-ox* feeding, with light phase in L/D conditions significantly lower than both dark phase and

midday-midnight. In L/L conditions TM feeding only shows % of total herbivory in s light phase as significantly lower than s dark phase. Bearing in mind differences in total feeding, changes in total feeding in (s) dark, (s) light and (s) midday-midnight periods are not widely different between the 3 genotypes. However, when comparing light and midday-midnight periods to dark phase, TM L/D feeding becomes significantly different from WT L/D. Normalizing against total feeding however shows no significant differences across genotypes. Additionally, when comparing CircaCompare results between the L/D and L/L conditions of *cca1-ox* and TM feeding no significant differences are found, shown in Suppl. Fig. 9.

Table 4.8 *P. xylostella* larval feeding on *A. thaliana* data.

Data from Fig. 4.5, larval *P. xylostella* feeding on varying *A. thaliana* genotypes and under L/D and L/L conditions, with ±SEM and significant difference groupings from statistical analysis. Pixel count loss from leaves is shown and the % of total pixel loss occurring within specific phases. The 12h periods matching (s) dark phase, (s) light phase and (s) midday-midnight were compared. Midday-midnight includes the 6 time points before and after dark phase onset. WT referring to Col-0 *A. thaliana* and TM to *cca1-11/lhy-21/toc1-21* triple mutant. * shows significant differences from dark phase equivalent, ^ shows significant differences from light phase equivalent. ✯ shows significant differences from the proportion of feeding in 1st ½ light phase compared to 2nd and significant linear regression slope of such.

Condition (n)	Total	Sig	(s)Dark	Sig	(s)Light	Sig	(s)Midday -Midnight	Sig	(s)Dark %	Sig	(s)Light %	Sig	(s)Midday-Midnight %	Sig	2nd ½ light %	Sig	Anticipation slope
WT L/D (12)	39092 ±2576	ac	21217 ±1931	ad	17875 ±1130	a	21040 ±1403^	a	53.09 ±2.166	n/a	46.48 ±2.288	n/a	53.73 ±1.301^	n/a	53.72 ±2.524	n/a	0.38*
WT L/L (8)	48439 ±4080	a	29970 ±3371	b	18469 ±1649*	a	26081 ±1838^	a	61.21 ±3.039	n/a	38.79 ±3.039*	n/a	54.34 ±1.371^	n/a	52.55 ±3.656	n/a	0.36*
CCA1-ox L/D (8)	11702 ±2211	b	6926 ±1437	c	4776 ±918.5	b	5625 ±899.1	b	57.61 ±3.778	n/a	42.39 ±3.778	n/a	49.88 ±2.202	n/a	44.86 ±3.129	n/a	-0.02
CCA1-ox L/L (9)	12377 ±1757	b	7014 ±908.0	c	5697 ±963.3*	bc	6898 ±915.4^	b	57.45 ±1.648	n/a	42.43 ±1.864*	n/a	52.9 ±1.885^	n/a	46.24 ±3.711	n/a	-0.11
TM L/D (8)	29548 ±1974	cd	16782 ±1245	de	12766 ±1145*	ad	14638 ±1081^	ab	56.79 ±2.294	n/a	43.21 ±2.294*	n/a	49.58 ±1.247^	n/a	45.13 ±2.431	n/a	-0.23*
TM L/L (8)	20656 ±3057	bd	11519 ±1772	cef	9137 ±1600	bde	10566 ±1512	ab	56.35 ±2.595	n/a	43.65 ±2.595*	n/a	51.94 ±2.341	n/a	49.42 ±2.895	n/a	-0.04
aos L/D (8)	42065 ±2746	a	25866 ±1601	ab	16198 ±1981*	af	21337 ±1906*	ac	62.17 ±2.801	n/a	37.83 ±2.801*	n/a	50.74 ±2.987*	n/a	45.62 ±3.384	n/a	0.03
aos L/L (8)	27337 ±2387	d	15704 ±1462	df	11632 ±1485	cd	14060 ±1447	ab	57.86 ±3.419	n/a	42.14 ±3.419	n/a	50.80 ±1.891	n/a	46.79 ±2.551	n/a	-0.13
opr3 L/D (8)	21342 ±2278	bd	12014 ±1631	cef	9714 ±637.0	bd	11308 ±1329	ab	54.90 ±2.146	n/a	47.24 ±2.489	n/a	52.91 ±2.251	n/a	51.78 ±2.789	n/a	0.22
opr3 L/L (7)	13997 ±1302	b	8124 ±616.1	cef	5873 ±795.5*	be	6510 ±559.6*	b	58.69 ±2.327	n/a	41.31 ±2.327*	n/a	47.35 ±3.206*	n/a	46.13 ±3.523	n/a	0.02
coi1-16 L/D (8)	17829 ±2473	bd	11065 ±1576	cef	6669 ±1261*	bd	8922 ±1451*	bc	62.38 ±3.525	n/a	37.62 ±3.525*	n/a	49.54 ±1.935*	n/a	48.12 ±2.177	n/a	0.13
coi1-16 L/L (7)	13784 ±2104	b	7573 ±1131	cf	6211 ±1275	be	7008 ±683.9	b	56.74 ±4.825	n/a	43.26 ±4.825	n/a	53.67 ±3.598	n/a	47.97 ±3.236	n/a	0.22

Similar changes in rhythmic features of *P. xylostella* larval feeding can also be seen when feeding on *A. thaliana* JA mutant leaves. *aos* mutant feeding shows somewhat comparable total feeding to WT *A. thaliana* compared to *cca1-ox* mutant, more similar to TM, but with *aos* L/D feeding being non-significantly different to both WT L/D and L/L feeding, shown in Fig. 4.5 and Table 4.8. *aos* L/D total feeding is significantly higher than all other mutants, however in L/L this is no longer the case, only higher than other JA mutant L/L conditions and *cca1-ox* shown in Table 4.7. Recorded *opr3* and *coi1-16* total feeding levels are not significantly different from each other or clock mutants, minus TM L/D from *opr3* and *coi1-16* L/L. JA mutant feeding (s) dark phase *aos* L/D, *opr3* L/L and *coi1-16* L/D show significantly more feeding than (s) light phase, while other conditions and mutants do not, with (s) midday-midnight also significantly lower than (s) dark phase. Similar to clock mutant results, no significant differences are found when comparing proportion of total light phase feeding occurring in either ½ of (s) light phase with no significant anticipation slopes. Comparing across genotypes in Table 4.8, there are again similar findings to clock gene mutants and after normalizing for differences in total feeding there are no significant differences found for (s) dark, (s) light and (s) dark phase onset. When comparing findings to WT *A. thaliana* CircaCompare results, *aos* has significantly increased rhythmic amplitude in L/D and significantly reduced in L/L. In L/D the phase peak is also significantly delayed, shown in Suppl. Table 2. *opr3* L/D results showed no significant differences to WT while L/L had significantly reduced rhythmic amplitude and delayed phase peak and *coi1-16* showed L/D results with significantly increased rhythmic amplitude and delayed phase peak, while L/L showed no significant differences. When comparing changes in the CircaCompare cosinusoidal curve fitting between JA mutant L/D and L/L results, only *aos* showed any significant differences with a significant reduction in rhythmic amplitude in L/L conditions, shown in Suppl. Fig. 9.

4.3.2.2 Col-0 and clock mutant *A. thaliana* choice assay

In choice assays comparing larval *P. xylostella* feeding on WT *A. thaliana* compared to clock mutant leaves, significant differences were found between % of feeding taking place on *cca1-ox* leaves. Shown in Fig. 4.6A, overall amount of leaf area loss was found to not be significantly different, yet, with ~80% of feeding taking place on WT *A. thaliana* leaves on average across the 8 tested time points, the increased feeding success between the two genotypes was significant.

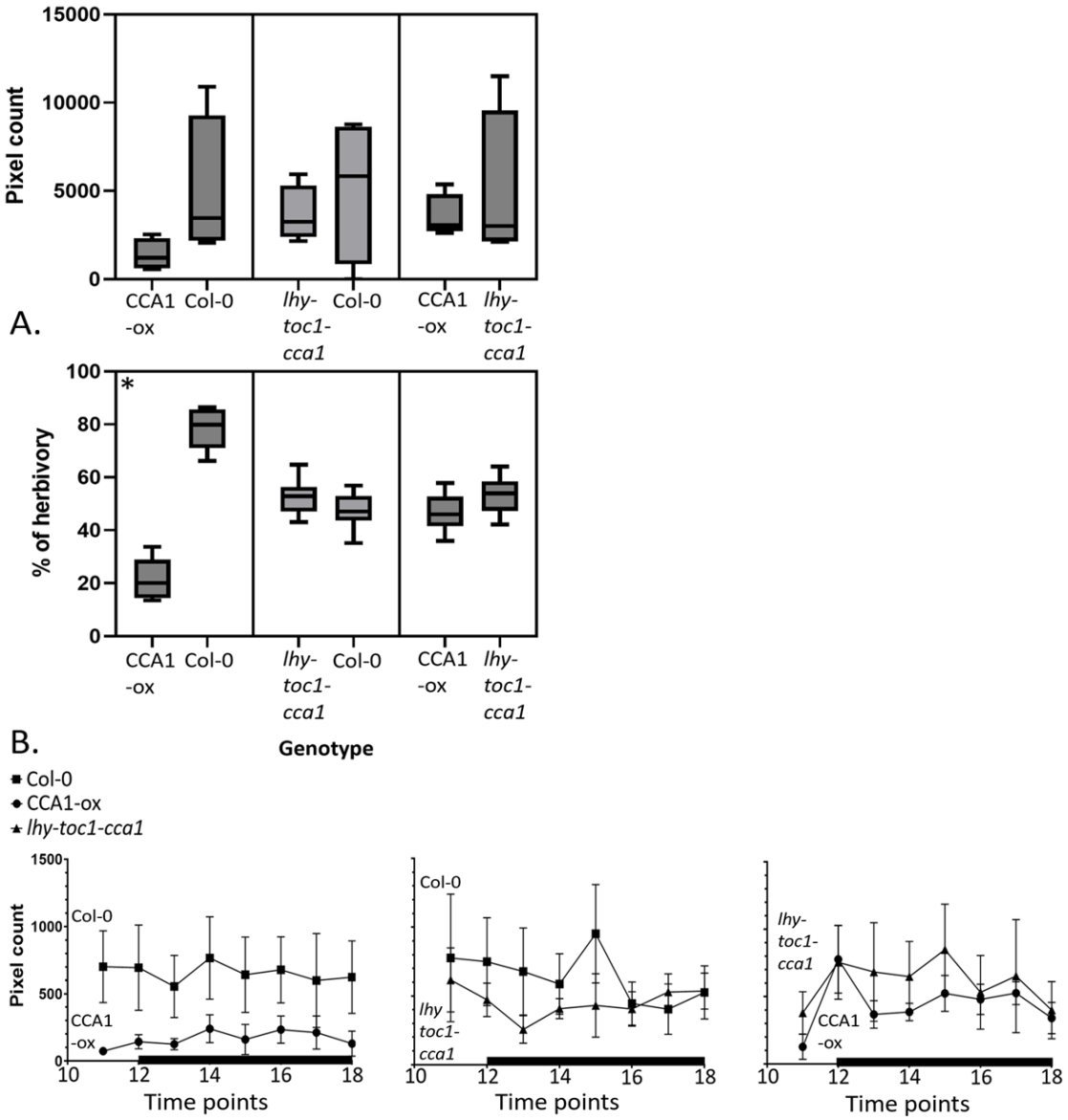


Figure 4.6 Choice assay between Col-0 and clock mutants.

Larval *P. xylostella* show significant difference for feeding on WT Col-0 *A. thaliana* compared to *cca1-ox* mutant in a choice assay, however, there isn't any clear differences in feeding choice between *cca1-lhy-toc1* triple mutant (TM) *A. thaliana* and WT or *cca1-ox* plants. A. Box and whisker plots with Tukey range (1.5xIQR) showing (top) total average pixel count area loss between genotypes of *A. thaliana* leaves and (bottom) average % of herbivory per time point taking place on *A. thaliana* leaves of a specific genotypes (n = 4). * shows significant difference between data. B. Larval *P. xylostella* feeding on *A. thaliana* pixel count loss over time in choice assay. Time points indicate zeitgeber time (ZT). Line graph with SEM error bars showing (left) feeding between WT Col-0 and *cca1-ox*, (middle) feeding between WT and *cca1-lhy-toc1* and (right) between *cca1-ox* and *cca1-lhy-toc1*. Black bar along x-axis signifying dark phase.

Other than the significant difference in WT and *cca1-ox* feeding choice, no differences in feeding preference between WT and TM are found. Moreover, when comparing TM and *cca1-ox*, though when compared to WT significantly less feeding occurred on *cca1-ox*, no significant differences were found between *cca1-ox* and TM. Fig. 4.6B shows how the differences in leaf area loss changed over the individual time points, however the variation in losses within a time point as well as repeat number being low, lead to no significant differences over time being found.

4.4 Discussion

4.4.1 Larval *P. xylostella* feeding rhythms

Larval *P. xylostella* demonstrated rhythmicity in feeding across the 24h day while feeding on *B. rapa* leaves. Fig. 4.1 shows presence of rhythmic feeding in numerous conditions, including in Fig. 4.5 on various genotypes of *A. thaliana* leaves. In Fig. 4.1 (left), feeding rhythms on IP *B. rapa* leaves in L/D are shown, which the stock colonies of *P. xylostella* have been reared on for generations in addition to the prior entrainment to the exact experimental set up before image collection began. A consistent trough in feeding activity appearing at (s) light phase onset in LD, L/L1 and L/L2 IP conditions shows a notable response to light, this also reflected by the early (s) dark phase peak of cosinusoidal fitting in these conditions. This trough entrained to light phase onset may be related to the sudden onset of bright light (~2700 lux) in these setups, as startle responses have been found in other species (Truman, 1974, Fenckova et al., 2019, Varnon and Adams, 2021), however, there isn't an abrupt change at this point, instead showing a decline towards and maintenance of the trough. It may be beneficial for *P. xylostella* to avoid feeding activity at time points between dawn and midday for numerous reasons. Foremost may be exposure to predators, including to both diurnal and crepuscular predators which may have increased feeding efficiency around dawn, with research also showing increased prey aversion responses during this time (Longcore and Rich, 2004, Kerckhove, Shutter and Abrams, 2012, Catano et al., 2017, Mcmunn et al., 2019). Other lepidopterans focus activity away from light phases being predominantly nocturnal (Kawahara et al., 2018), allowing for increased survival and avoidance of predators which can be a selection pressure for nocturnality (Daily and Ehrlich, 1996, Gordan, Dickman and Thompson, 2010). Other lepidopteran pests also avoid feeding during the light phase, such as *Spodoptera litura,* avoiding biotic stressors by burrowing into soil during the day, also helping to avoid

abiotic stresses (Zhang et al., 2021). Further stresses can occur in the morning that may significantly affect *P. xylostella* larvae. Humidity significantly effects *P. xylostella* development and survival rates with irrigation practises also being shown to be effective control methods (Lim, 1982, Lee and Huang, 1986, Guo and Qin et al., 2010, Machekano et al., 2019, Farias et al., 2020). Humidity often peaks in the morning (Mwimba et al., 2018, National Weather Service, 2022), which may inhibit feeding and *P. xylostella* survival in natural conditions, leading to endogenous rhythms in feeding that avoid this time. Artificial stressors in addition to irrigation techniques that have been shown to affect *P. xylostella* is the use of chemical control treatments such as pesticides, which may have led to wild populations of *P. xylostella* preferentially laying eggs closer to the base of crops (Sarfraz, Dosdall and Keddie, 2005). Pesticide treatments are often sprayed first onto crops in mornings and towards midday (Hamka et al., 2021), however in surveys, though the majority of farmers surveyed consider the timing of spraying, only ~1/3 specifically timed when they used pesticides (Kafle et al., 2021). These usual pesticides application regimes match the recorded *P. xylostella* larval feeding trough period, which may be related to selection via such agricultural control techniques before the used *P. xylostella* strain was collected from the wild. Natural plant defences can also be under the control of circadian clocks that control the time-dependent expression of plant defences (Goodspeed et al., 2012, Pieterse et al., 2012, Goodspeed et al., 2013, Thines, Parlan and Fulton, 2019). Herbivory defences such as glucosinolates are upregulated during the day, peaking between light phase onset and midday (Goodspeed et al., 2013b), also matching the observed *P. xylostella* feeding trough period, demonstrating a possible avoidance behaviour of increased plant herbivory defences. It is evident that feeding during light phase may have many drawbacks to *P. xylostella,* which may explain why across the results in this chapter feeding is often significantly higher in (s) dark phase conditions.

In all IP conditions minus L/L1, there was a significantly lower proportion of feeding occurring across light phase compared to (s) dark phase. As mentioned previously, the majority of lepidopteran species are nocturnal, excluding the Rhopalocera sub-order (Butterflies) (Kawahara et al., 2018), therefore in addition to previous research on *P. xylostella* showing dark phase activity preference (Pivnick et al., 1990, Talekar et al., 1994), this demonstrates *P. xylostella* larvae also being somewhat nocturnal. During extending L/L conditions, feeding was also reduced with IP L/L2 and L/L3 having significantly reduced total feeding compared to IP L/D. Though nocturnal feeding is likely beneficial via avoiding the light phase associated threats discussed previously, *P. xylostella* larvae show additional features to their rhythmic feeding profiles.

Demonstrated by cosine curve fitting, the phase peak of feeding across all light conditions is within the (s) dark phase, however, compared to IP L/D and L/L2, L/L1 is significantly advanced towards s dark phase onset with L/L3 delayed towards the centre of s dark phase. These results demonstrate increased activity around the end of light phase/(s) dark phase onset, shown also in Fig. 4.1 and 4.2. The conditions maintained in larval feeding assays were constant apart from the provision of lights on and off at consistent times across the 24h day in L/D conditions. Before these abrupt changes in abiotic conditions *P. xylostella* larval feeding changes. Anticipation of changes in environmental conditions allows for the regulation of physiological mechanisms to best suit changing environments, maintaining fitness by preparing for such changes (Paranjpe and Sharma, 2005, Vaze and Sharma, 2013, Xu et al., 2022). As mentioned previously, before (s) light phase onset, (s) dark phase feeding declines over time, leading to phase peaks nearing (s) dark phase onset in IP conditions, demonstrating possible anticipation of lights on, where still active *P. xylostella* larvae may be more exposed to threats. Similar anticipation is clearer towards the end of light phase where there is a significant increase in feeding with significant slopes of increasing proportions of total herbivory towards (s) dark phase onset in IP L/D and L/L1

(including in *A. thaliana*). This (s) dark phase anticipation is also evident in IP by significantly higher proportion of (s) light phase feeding occurring in the second half of such, shown in Table 4.1. The proportion of feeding 6h before and 6h after s dark phase onset (midday-midnight) therefore has significantly more feeding compared to s dark phase in IP L/L1 conditions. Increased feeding before dark phase onset may be beneficial as though there is still light, lowerd light conditions may reduce predator efficiency, though still selecting for more mobile species more capable of avoiding predators (Clarke, 1983, Thomas and Jacobs, 2013, Kirkeby, Wellenreuther and Brydegaard, 2016), while high temperatures allow for increased insect activity (Mellanby and Gardiner, 1939, Schou et al., 2013, Sinclair et al., 2016). This can be shown across insect communities as an evening insect activity peak (Kirkeby, Wellenreuther and Brydegaard, 2016). *P. xylostella* host plant defences may also be lowerd at this time with *A. thaliana* and *Brassica oleracea* (another common *Brassica* crop species) both showing low glucosinolate and JA levels near this time (Goodspeed et al., 2012, Goodspeed et al., 2013a,b). Similar anticipatory effects are shown with *D. melanogaster*, which anticipates both light and dark phase onset, showing crepuscular activity rhythms (Tataroglu and Emery, 2014, Dubowy and Sehgal, 2017). The anticipation of these environmental changes likely offers *P. xylostella* larvae increased fitness as shown in other organisms (Paranjpe and Sharma, 2005, Vaze and Sharma, 2013, Xu et al., 2022). Lepidopteran species show ‘well-timed’ activity, including egg hatching in *Bombyx mori* (Domestic silk moth) that show morning anticipation (Minis and Pittendrigh, 1968, Sakamoto and Shimizu, 1994). Larval feeding also has been shown to occur rhythmically, albeit not consistently across all species (Reynolds and Timmins, 1986, Bernays and Singer, 1998, Kim et al., 2011, Suszczynska et al., 2017, Zhang et al 2021), though arrhythmic species’ behaviours may express weak rhythms in response to certain zeitgebers e.g. with *D. plexippus* and *Chloridea virescens* (Tobacco budworm)

(Niepoth et al., 2018). The results from this chapter show that *P. xylostella* larval feeding is rhythmic, at least in response to the experimental setups and prior entrainment conditions.

However, as L/L pre-entrainment conditions are maintained for longer periods before experiment start, changes in the rhythmic features of *P. xylostella* larval feeding occur. In constant conditions such as D/D, endogenous free-running circadian rhythms can be recorded such as with *D. melanogaster* and certain lepidopteran species (Minis and Pittendrigh, 1968, Giebultowicz et al., 1989, Goodspeed et al., 2012, Shindey et al., 2016, Dubowy and Sehgal, 2017, Zhang et al., 2021). Such rhythms can also be maintained in L/L, however, the effect of light on the molecular circadian clock can lead to shifts in rhythmic features and the rundown of circadian rhythms, leading to arrhythmic results (Possidente and Birnbaum, 1979, Marrus, Zeng and Rosbash, 1996, Yoshii et al., 2005, Allada and Chung, 2010, Shindey et al., 2016). Recorded *P. xylostella* larval feeding demonstrates maintenance of rhythmicity into L/L conditions, shown in Fig. 4.1 and 4.2, with rhythmic data recorded in L/L3 conditions shown in Table 4.2. However, rhythmic amplitude decreases significantly towards this length of time in L/L, with the anticipation of (s) dark phase onset lost, noted by changes in phase peak, distribution of feeding activity and anticipation slope shown in Table 4.1. These gradual changes show evidence of the rundown of a circadian clock that would otherwise be involved in maintaining the rhythmic activity seen in previous conditions. Phase anticipation also demonstrates evidence of an active circadian clock used in the control of the recorded behaviour (Stoleru et al., 2004). Additionally, *P. xylostella* larval feeding maintained anticipation in L/L1 with somewhat increased s dark phase onset anticipation as the proportion of feeding activity within s midday-midnight is shown to be significantly higher than average (s) dark phase feeding, which is non-significantly different in L/D. The presence of an endogenous rhythm that is rundown in L/L is comparable to previous results found from species with active molecular circadian clocks (Marrus, Zeng and Rosbash, 1996, Emery et al., 2000,

Chen et al., 2008, Allada and Chung, 2010, Helfrich-Forster, 2020), demonstrating that *P. xylostella* larval feeding is likely under circadian regulation, though impacted by light cycle conditions.

4.4.2 Out-of-phase larval *P. xylostella* feeding rhythms

P. xylostella larvae fed using OP *B. rapa* leaves, maintained using an opposite L/D cycle (12h advanced), produced differing rhythmic feeding profiles to *P. xylostella* fed using *B. rapa* leaves that were entrained to the same light cycle. OP feeding profiles are shown in Fig. 4.1 (right) and show similarities and differences to IP feeding. In OP L/D conditions, (s) dark phase feeding was significantly higher than light phase as both leaf area loss and as proportions of total feeding. This is similar to results from IP L/D counterparts except losing increased feeding near the end of (s) light phase/(s) dark phase onset, showing a loss of (s) dark phase anticipation. Neither (s) dark phase onset as a proportion or as total feeding is significantly more than (s) light phase results, moreover, (s) dark phase onset as a proportion of total feeding is significantly lower than (s) dark phase. In addition to no significant differences between each ½ of light phase feeding or significant anticipation slopes, these findings show a clear distinction between (s) dark and (s) light phase feeding behaviours compared to IP, being specifically high in (s) dark and low in (s) light. Nocturnal activity rhythms in other *P. xylostella* findings are conserved, however a concentration of activity soon after (s) dark phase onset is also lost (Pivnick et al., 1990, Talekar et al., 1994 Lee, Lee and Boo, 2005), showing a maintenance of high feeding activity across the (s) dark phase. The advantages of dark phase feeding by *P. xylostella* have been previously outlined (in 4.4.1), therefore it may be beneficial for *P. xylostella* to maintain high feeding across the dark phase to maximise fitness. These OP rhythmic feeding profiles show phase peaks around the middle of (s) dark phase as a consequence of this, significantly delayed compared to IP feeding.

In general, increasing pre-exposure to L/L conditions doesn't affect the OP feeding rhythms found, though becoming arrhythmic by L/L3, around when phase anticipation is lost in IP feeding, with no significant differences in amplitude and phase peak across conditions. Though OP L/L2 has significantly higher s dark phase onset feeding than light phase, this is still significantly lower than the total proportion in s dark phase. The loss of dark phase therefore appears to have a more minimal impact on the feeding rhythms of *P. xylostella* when exposed to OP *B. rapa* leaf tissues compared to IP. This may be due to the loss of more complex feeding rhythms via the loss of entrainment with host plant leaf tissue rhythms, which volatiles from such may inform *P. xylostella* behaviour (Pivnick et al., 1990, Miranda-Anaya, Guevara-Fefer and Garcia-Rivera, 2002, Campos, Schoereder and Sperber, 2004, Campos, 2008), also leading to the significantly lower rhythmic amplitudes recorded. Previous research with a generalist insect herbivore (*T. ni*) showed both maintenance of larval feeding rhythms into constant conditions (D/D) and showed a significant increase in larval weight gain and host plant tissue loss with OP feeding compared to IP (Goodspeed et al., 2012, Goodspeed et al., 2013b). *P. xylostella*, a specialist brassica herbivore, also showed maintenance of larval feeding rhythms into constant conditions, however, these were significantly altered by OP entrainment of host plant leaves and didn't show significantly increased feeding. Feeding around OP L/D (s) dark phase onset was significantly reduced compared to IP, however, this shows change in rhythmic feeding profile rarther than total feeding. These findings show evidence that circadian clock regulation endogenous to *P. xylostella* maintains nocturnal peak feeding activity in both IP and OP constant conditions, however, also interacts with the circadian regulation of host plants, defining changes in rhythmic and anticipatory feeding activity.

4.4.3 Interaction with the host plant circadian clock

Differences between results from the conditions tested in Fig. 4.1 and findings from *T. ni* may be due to species-specific interactions between *P. xylostella* and *B. rapa* host plants. It is evident from IP *P. xylostella* larval feeding profiles that circadian rhythms in feeding are present, in addition to such being under the control of a circadian clock that is rundown in continued L/L. It is somewhat unclear if these rhythms are provided by the host plant or pest circadian clocks however. Though animal circadian clocks have been shown to damp out in L/L and loose rhythmicity (Marrus, Zeng and Rosbash, 1996, Emery et al., 2000, Chen et al., 2008, Allada and Chung, 2010, Helfrich-Forster, 2020), plant circadian clocks have been shown to be more robust, maintaining rhythmicity into L/L conditions (Hicks et al., 1996, Thain et al., 2002, Oakenfull and Davis, 2017). However, recent research using CAB:LUC reporter system has shown a loss in certain plant metabolite rhythms after ~4 days in L/L (Millar et al., 1995, Thain et al., 2002), similar to when *P. xylostella* larval feeding rhythms appear to loose rhythmicity also. It is evident however from OP feeding results that IP rhythms are not solely reliant on plant circadian rhythms, due to there not being a 12h shift in phase or maintenance of all rhythmic features. Instead, results suggest an interaction between host plant and pest circadian clock entrainments.

There may not be increased total feeding in OP *P. xylostella* feeding due to the 12h shift in host plant defence circadian rhythms. *P. xylostella* has been shown to be dependent on the detoxification of glucosinolates by GSS for feeding on brassicas (Li et al., 2000), and due to the 12h phase shift in plant clock, peak herbivory defences now occur during *P. xylostella* IP and OP maintained peak feeding times instead of during the trough (Goodspeed et al., 2012, Goodspeed et al., 2013b). This shift in defence regulation may allow generalist pests, and potentially other diurnal pests such as *T. ni*, to increase their feeding due to herbivory defences being expressed during the (s) dark phase (Goodspeed et al., 2012, Goodspeed et al., 2013b). For *P. xylostella* however, as a specialist nocturnally

active pest, potentially reliant on the production of specialised detoxification proteins (GSS), this shift in herbivory defences may not only reduce potentially increased feeding levels due to OP conditions, but also mute rhythmic features such as feeding increases towards (s) dark phase onset (away from peak glucosinolate) and deceased feeding towards light phase onset (towards peak glucosinolate). Consequently leading to reduced rhythmic amplitude and rhythmicity.

The IP *P. xylostella* feeding rhythm matches the inverse pattern of glucosinolate concentration in *B. oleracea* closely (Goodspeed et al., 2013b). As previously mentioned, due to the products produced from glucosinolate in the MOB being toxic to *P. xylostella,* the found feeding rhythms may be in response to herbivory deterrent volatile signals from the plant or detected in the *P. xylostella* gut after feeding. Similar pathways have been shown to affect behaviour in previous research (Pivnick et al., 1990, Campos, Schoereder and Sperber, 2004, Yan et al., 2014, Li et al., 2018). This would demonstrate that *P. xylostella* feeding rhythms can be dictated by the regulation of plant circadian clocks, yet, the maintenance of (s) dark phase focused feeding activity shows that there is maintenance of an entrained endogenous rhythm in *P. xylostella* larvae that doesn't respond immediately to shifts in host plant volatile signals, as there is no evidence of re-entrainment in OP conditions. *P. xylostella* when entrained with *B. rapa* may feed in time with light cycles and glucosinolate presence, sensing such through olfactory receptors (Yan et al., 2014, Li et al., 2018), however, when out of sync with host plant tissues may attempt to carry out feeding in the comparative safety of dark phase, though leading to somewhat restricted feeding due to increased levels of herbivory deterrents during *P. xylostella* active phase (Goodspeed et al., 2013b).

4.4.4 *P. xylostella* clock and GSS gene expression rhythms

4.4.4.1 Circadian clock gene expression

The core molecular circadian clock genes *per* and *tim* were found to be expressed rhythmically in larval *P. xylostella* fed on IP and OP *B. rapa* leaves, shown in Fig. 4.3, peaking during the middle of (s) dark phase with the trough in the middle of light phase. This demonstrates evidence for the presence of an active circadian clock, which may regulate herbivory rhythms. *per* and *tim* are crucial parts of the circadian clock and their rhythms of expression shown through qPCR in this chapter show circadian clock genes that undergo ~24h cycles (Marrus, Zeng and Rosbash, 1996, Rosato, Tauber and Kyriacou, 2006), with *per* being shown to be important for maintenance of anticipatory behaviours (Grima et al., 2004). In addition, the expression rhythms of such are maintained into L/L, which not all previous research on lepidopterans have shown (Merlin, Gegear and Reppert, 2009, Kobelkova et al., 2015, Zhang et al., 2021). The precise expression rhythms are similar however to both some diurnal and nocturnal lepidopteran species such as in *D. plexippus, B. mori* and *H. armigera* (Merlin, Gegear and Reppert, 2009, Kobelkova et al., 2015, Yan et al., 2017, Ikeda et al., 2019, Zhang et al., 2021). Though rhythms are maintained, there are notable shifts in relative expression with *per* and *tim* rhythmic amplitude decreased in both IP L/L conditions and OP L/L *per* relative expression. OP *tim* relative rhythmic amplitude remains unchanged however the mesor is increased and phase peak advanced, with *per* rhythmic amplitude significantly different between IP and OP conditions. The overall relative expression rhythms of clock genes in both IP and OP remain relatively unchanged however, demonstrating a maintenance of circadian clock function on OP host plant material compared to IP. These findings show that *P. xylostella* endogenous rhythms are likely maintained via active cycling of molecular circadian clock, demonstrating a pathway for the regulation of circadian rhythms in *P. xylostella* physiology. These rhythms are also potentially robust due to minimal changes in *per* and

tim expression in L/L conditions, with similar shown in *H. armigera* (Cotton bollworm) D/D (Yan et al., 2017), compared to other tested lepidopterans (Merlin, Gegear and Reppert, 2009, Kobelkova et al., 2015, Zhang et al., 2021). These previously tested Lepidoptera, including *D. plexippus, B. mori* and *S. litura*, show significant changes in relative expression of *per* and *tim* in constant conditions, with more minimal changes occurring in *P. xylostella* L/L results by comparison, similar to the slight variation over circadian time shown in *P. xylostella* feeding rhythms. When exposing *P. xylostella* to OP plants, the maintenance of *P. xylostella* circadian clock appears to be unchanged by any shifts in plant volatile signals, demonstrating that the upstream circadian regulation of *P. xylostella* physiology, including that related to feeding, remains unchanged. However, it is important to consider how peripheral clocks, such as those in the gut which can often be independently entrained, may respond with previous research showing CCN (Central clock neurons) independent regulation and entrainment (Sauman and Reppert, 1998, Xu, Zheng and Sehgal, 2008, Nobata et al., 2012, Zhang et al., 2021).

4.4.4.2 GSS gene expression

The relative expression of GSS genes however would potentially be more likely to respond to changes in host plant volatile rhythms due to their expression in the midgut and role in disarming the MOB (Ma et al., 2018, Heidel-Fischer et al., 2019), however the relative expression profiles found show possible regulation via pest and host circadian clocks and interactions with L/D light cycle. *Sulf2* and *Sulf3* expression showed significant changes over time, though often didn't show significant rhythmicity across experimental conditions, shown in Fig. 4.3 and Table 4.3. IP GSS genes showed no specific time points being significantly different from each other, however, L/L *Sulf2* expression was shown to have significant rhythmicity with significant change over time in L/D and L/L. *Sulf2* expression is shown to be important for universal glucosinolate interactions (minus root specific glucosinolate), being the most highly expressed across *P. xylostella* larval life

stages and organs (Ma et al., 2018, Heidel-Fischer et al., 2019), potentially identifying *Sulf2* as the most important GSS gene. The expression of *Sulf2* in larval life stages and the midgut (also identified to be highly expressed in the salivary glands and malpighian tubules) (Ma et al., 2018) may be rhythmically expressed to match or be entrained by the presence of glucosinolate in the *P. xylostella* larval midgut after feeding or through olfactory signals (Yan et al., 2014, Li et al., 2018, Heidel-Fischer et al., 2019), originating from plant hosts. As feeding is high when glucosinolate concentration is potentially low in *B. rapa* and glucosinolate concentration peaks when feeding is low (Goodspeed et al., 2013b), the exposure to glucosinolate in the gut of *P. xylostella* larvae may even out over the 24h day, leading to the non-significant rhythmicity in GSS gene expression recorded. Additionally, previous research has shown that *Sulf2* is inducible by the presence of glucosinolates (specifically Met glucosinolate) (Heidel-Fischer et al., 2019). For *Sulf2* IP L/L relative expression, the phase peak was recorded at 23.67h from s dark phase onset, showing a peak in expression rhythms in L/L within 1h of the *B. rapa* feeding phase peak in these conditions. *Sulf3* was shown to interact with a specific subset of glucosinolate varieties, specifically lacking interactions with Phe or Trp based glucosinolates which includes indole and benzenic glucosinolates, which have a lower proportional presence in leaf tissues (Hopkins, Dam and Loon, 2009, Sonderby et al., 2010, Ishida et al., 2014, Heidel-Fischer et al., 2019). Potentially demonstrating *Sulf3* as an additional GSS gene for reacting with the higher levels of aliphatic glucosinolate in leaf tissues *P. xylostella* may consume. *Sulf3* is expressed to a similar level of *Sulf2* in IP conditions and is likely important for maintaining the disarmament of the MOB during *P. xylostella* feeding (Ma et al., 2018, Heidel-Fischer et al., 2019). Similar to *Sulf2* expression, *Sulf3* is shown to be expressed across time in IP L/D and L/L conditions with no significant differences between conditions or times points, lacking significant rhythms. Though in both GSS genes, 2-way ANOVA shows that a significant portion of the recorded variation occurs due

to changes across time points. These results show a potential interaction between both *P. xylostella* feeding activity and regulation of plant defences for the regulation of GSS genes.

OP condition results show these expression rhythms are likely a result of interactions with the host plant clocks and defences and maintenance of endogenous *P. xylostella* circadian clock regulation. Between OP and IP conditions, where the regulation of plant herbivory defences are phase shifted by 12h, no notable significant differences between *Sulf2* expression were found, also shown in Table 4.3. Bearing in mind *Sulf2*'s universal interaction with glucosinolates and inducibility by subsets of such (Heidel-Fischer et al., 2019), this shows glucosinolate presence had a somewhat minimal effect on GSS gene regulation. OP *Sulf2* relative expression levels in L/D conditions show both significant rhythmicity and changes over time between individual time points, with a significant decline from ZT13 to ZT21, then climbing to ZT5 with both ZT13 and ZT5 showing peak expression levels. This shows a rhythm somewhat similar to peak feeding in IP conditions and glucosinolate concentration in brassicas (Goodspeed et al., 2013b). These changes over time are mostly lost however in L/L conditions with a significant advancement in cosinusoidal curve phase peak, occurring around s dark phase onset. No significant differences were also found between *Sulf2* IP and OP relative expression, showing the shift in host plant circadian regulation had minimal effect on the expression of such, with *P. xylostella* CCN entrainment likely maintaining regulatory control or through midgut peripheral clocks (Sauman and Reppert, 1998, Xu, Zheng and Sehgal, 2008, Nobata et al., 2012, Zhang et al., 2021). The relative expression of *Sulf3* showed many significant differences between IP and L/D-L/L conditions. Circadian rhythms could not be compared directly between IP and OP conditions as IP *Sulf3* expression lacked rhythmicity, however, significantly different relative expression levels across both L/D and L/L days were shown. More notable is the shift in rhythmic phases between OP L/D and L/L conditions, with OP *Sulf3* expression having significant rhythmicity compared to IP. L/D OP *Sulf3* expression

shows a phase peak in early light phase, similar to the trough in feeding during IP feeding. This phase peak is not significantly different from that of OP *Sulf2* but has a significantly increased mesor and rhythmic amplitude, demonstrating greater overall expression and changes in such. This may be in response to the unanticipated high levels of glucosinolate accumulating in the *P. xylostella* midgut due to high (s) dark phase feeding in OP conditions with peak glucosinolate concentration occurring during dark phase on OP *B. rapa* leaves, instead of during light phase (Goodspeed et al., 2013b). This showing that certain GSS genes (*Sulf3*) may respond more to changes in host plant volatiles compared to others (Sulf2). There appears to also be an interaction with light cycles as the *Sulf2* ZT17 peak in IP conditions appears to be shifted to ZT13 in OP conditions, however in both L/L conditions there appears to be less difference between the two profiles. The glucosinolate varieties *Sulf3* is specialised to interact with make up a higher proportion of overall glucosinolate content in leaf tissues (Hopkins, Dam and Loon, 2009, Sonderby et al., 2010, Ishida et al., 2014) *P. xylostella* preferential feed on (Ram et al., 2016), which GSS genes may be induced by (Heidel-Fischer et al., 2019) which may lead to the results found. The reasons for the loss of dark phase in L/L causing such a large shift in rhythms (by nearly 12h in phase peak) is unclear but demonstrates another interaction with the preovison of light cycles during *P. xylostella* larval feeding, however, OP *Sulf2* L/L rhythms are not significantly different to *Sulf3* in these conditions, only showing reduced rhythmic amplitude demonstrating maintenance of similar expression rhythms between the two in these conditions. The significant changes in GSS gene expression and rhythms between OP and IP *B. rapa* fed *P. xylostella* larvae show that the regulation of GSS genes is likely maintained via *P. xylostella* circadian rhythms, however interactions with plant defences and L/D cycles are present, specifically with *Sulf3* expression. Similar interactions with L/D cycles have been seen in *D. melanogaster* where circadian clock regulated genes that maintain rhythmic expression into D/D had higher rhythmic amplitude under L/D cycles

(Wijnen et al., 2006). The found *Sulf2* relative expression results are unlikely to be caused by coincidental responses to L/D cycles and host plant clocks however due to the similar expression profiles of GSS genes in OP L/L conditions to IP L/L. Similar cannot be said for *Sulf3* OP results further highlighting a potential different regulatory system. The lack of change in GSS regulation, specifically that of *Sulf2*, in response to OP conditions may also be responsible for the lack of increased feeding in OP conditions compared to findings from *T. ni* (Goodspeed et al., 2012, Goodspeed et al., 2013b), as may limit *P. xylostella*'s ability to outcompete the MOB defence via appropriately timed glucosinolate sulfatase expression.

4.4.5 *P. xylostella* larval weight gain

The results from larval weight gain show similarities and differences to feeding rhythms in IP and OP conditions. Weight gain over time is likely not only dependent on the quantity of larval *P. xylostella* feeding but through the expulsion of frass/faeces (defecation), which has been shown to occur rhythmically in *S. litura*, peaking during late light phase and ~12h from peak feeding (Zhang et al., 2021). Rhythmic defecation, matching feeding rhythms, has also been shown in other lepidopteran species such as *Lymantria dispar* (Gypsy moth) (Liebhold and Elkinton, 1988). Rhythmicity in *P. xylostella* larval weight gain was not found over L/D and L/L conditions from feeding on both IP and OP *B. rapa* leaves. In IP L/D conditions however, notable peaks in weight gain are shown at ZT16 and ZT12 during IP peak feeding with ZT12 leading into another peak weight gain point at CT16, following a similar trend to that seen in Fig. 4.1 IP feeding. In addition, there is potential increases in weight gain around ZT4, CT24 and CT28, similar to the time during low *P. xylostella* larval feeding, however these time points show high variation and are not significantly different to any other points across the time course. This variation may arise due to changes in defecation activity or pupal gut purging around these time points. Gut purging

as larval *P. xylostella* prepare for pupal life stages may have occurred, as only later instar *P. xylostella* larvae were used in experiments, potentially affecting weight gain measurements at certain times of day, with evidence of circadian control of such in *Samia Cynthia* (Mizoguchi and Ishizaki, 1982).

OP weight gain showed non-significantly different proportional weight gain to IP, apart from CT20 having significantly higher weight gain in OP conditions. Significantly higher weight gain was found at certain OP time points, often found in the (s) dark phase, again matching feeding rhythms found in OP *B. rapa* feeding. Though overall variation was high, reducing the number of significant differences, weight gain tended to match features of *P. xylostella* rhythmic feeding in both IP and OP conditions. Though weight gain may not directly relate to leaf area loss from potential crop plants, it can demonstrate the speed at which *P. xylostella* can develop, with increased weight reducing development time in other lepidopterans (Frago and Bauce, 2014), in addition to what proportion of plant tissue is being excreted compared to absorbed (Panizzi, Oliveira and Silva, 2004). There was also limited changes in total weight gain on OP *B. rapa* leaves compared to IP demonstrating limitations in any benefits to feeding on OP host plants in *P. xylostella* compared to the results shown in *T. ni* which showed significantly increased weight gain (Goodspeed et al., 2013b). These results may also backup leaf area loss findings, though due to high variation, which may arise from varaitions in defecation, this is unclear.

4.4.6 Larval *P. xylostella* feeding rhythms on varying *A. thaliana* genotypes

There was maintenance of similar rhythmic *P. xylostella* larval feeding patterns on WT *A. thaliana* with notable increases in feeding during midday-midnight in L/D conditions and significant linear regression slopes of increase in proportion of total feeding occurring from (s) midday to (s) dark phase onset (Anticipation slope) shown in Fig. 4.6 and Table 4.8. *A. thaliana* are within *P. xylostella*'s typical host plant family, producing a similar rhythmic

profile of glucosinolate concentration over time (Goodspeed et al., 2013b), potentially leading to the similar feeding rhythms recorded. However, in L/L there was a significant reduction in total feeding in the s light phase compared to the s dark phase, though feeding between s midday-midnight was still significantly higher than overall s light phase feeding. Both L/L and L/D showed no significant differences between proportion of feeding in the 2nd half of (s) light phase compared to 1st, however showed significant anticipation slopes, demonstrating maintenance of increases in feeding towards (s) dark phase onset, similar to *B. rapa* results. These findings, in addition to cosinusoidal curve fitting, demonstrated maintenance of *P. xylostella* larval *B. rapa* feeding rhythms on *A. thaliana*, albeit with significantly altered rhythmic features. In *A. thaliana* L/D compared to *B. rapa*, the rhythmic amplitude was significantly reduced while the phase peak was significantly delayed in L/L, towards more s dark phase focused feeding. The loss of a light cycle appeared to affect the maintenance of phase peaks near s dark phase onset further on *A. thaliana* further compared to *B. rapa*. The tested strain of *P. xylostella*, sourced from the Rothamsted Research and collected in 1960s, has been continuously maintained on *B. rapa* cultures since collection from the wild. Adaptation to these rearing conditions may have occurred, as has been recorded in many other herbivorous insects and with lab/artificial diet-reared moths (Grayson et al., 2015, Hoffmann and Ross, 2018). This may have led the used strain of *P. xylostella* to develop physiological responses adapted to *B. rapa* hosts plants after nearly a century of *B. rapa* interactions (Wybouw et al., 2015), potentially leading to a clearer recorded feeding rhythm when interacting with such hosts compared to novel hosts such as *A. thaliana*, where entrainment to such only occurred within a generation. Similar effects have been recorded in *Tetranychus urticae* (Red spider mite) which exhibited significantly increased transcriptional responses when re-exposed to host plants the strain had been maintained on previously (Wybouw et al., 2015). Short entrainment periods are important to consider however due to *P. xylostella*'s rapid

migration and selection of numerous brassica hosts, in addition to potential novel host range expansions (Harcourt, 1957, Chapman et al., 2002, Knolhoff and Heckel, 2011, Zalucki et al., 2012).

4.4.6.1 Effect of *A. thalaian* clock mutants

P. xylostella feeding on *A. thaliana* clock mutant leaves showed significant changes to feeding and rhythms in such. In L/D conditions *cca1-ox* feeding showed significantly delayed phase peak, peaking around the middle of (s) dark phase. This demonstrated results similar to OP *B. rapa* feeding where the plant clock was phase shifted by 12h compared to *P. xylostella,* wih upregulation of host plant defences during (s) dark phase. L/L conditions, where the (s) midday-midnight feeding total was reduced in WT, *cca1-ox* feeding showed no significant phase differences, though showed significantly reduced amplitude. Though rhythmic feeding was maintained on *cca1-ox* leaves, (s) phase onset peaks and troughs were lost compared to WT *A. thaliana* and *B. rapa,* loosing significant anticipation slopes, resembling effects of OP *B. rapa* feeding. This may be due to increased level of herbivory defences occurring during the (s) dark phase compared to WT, as is expected between IP and OP *B. rapa* (Goodspeed et al., 2012, Goodspeed et al., 2013b). *cca1-ox A. thaliana* mutants produce arrhythmic plants with significant increases in the regulation of certain defence pathways. In general, plant defences are increased due to the phase *cca1-ox* circadian regulatory mechanisms are locked in, however certain pathogens and generalist herbivores may have increased success on *cca1-ox A. thaliana* (Wang and Tobin, 1998, Goodspeed et al., 2013a, Lei et al., 2019, Zhang et al., 2019, Yamaura et al., 2020). As the plant clock is locked into a morning-like phase (upregulating PRR (Pseudo-response regulators) morning genes) (Matsushika et al., 2002, Nagel et al., 2015), upregulation of JA and herbivory deterrents occur, high glucosinolate levels are therefore expected across the 24h day (Brader, E and Palva, 2001, Goodspeed et al., 2013a,b, Kastell et al., 2013, Zhang et al., 2018, Lei et al., 2019). This may have caused

the similar rhythms found compared to OP *B. rapa* feeding due to high levels of plant herbivory deterrents during (s) dark phase. In addition to glucosinolates, other defensive molecules *P. xylostella* GSS genes do not interact with may be up regulated, including myrosinase which may compete with GSSs ability to interact with glucosinolate before MOB reactions can occur (Li et al., 2000, Ratzka et al., 2002). This, in combination with lowered nutritional quality and defensive phenotypes such as increased trichomes (Green et al., 2002, Traw and Bergelson, 2003, An et al., 2011), may have led to the significantly reduced *P. xylostella* larval feeding on *cca1-ox* leaves. Therefore, both the loss of rhythmic host plant clock function and the phase such is arrested in may affect *P. xylostella* larval feeding.

A. thaliana TM leaves lack expression of functional core circadian clock genes, leading to weakly rhythmic (responding to last end of dark phase) and arrhythmic plant tissues in L/L, with minimal circadian clock related regulation, lacking the function of the 3 core molecular plant clock components (McClung, 2006, Ding et al., 2007). This may have led to low levels of herbivory defence pathway regulation across the 24h day due to the lack of the clock-controlled JA regulatory pathways discussed previously. Loss of rhythms in such may have affected *P. xylostella* feeding also, leading to the lack of anticipatory feeding behaviours. TM L/D total feeding is significantly lower than WT L/L, with TM L/L significantly lower than both WT conditions. These changes may occur due to the lower levels of glucosinolate in arrhythmic plant tissues having reduced *P. xylostella* larval attraction (Yan et al., 2014, Li et al., 2018), along with reduced nutritional quality due to reduced fitness in arrhythmic plants (Green et al., 2002, Dodd et al., 2005). This further shows that both the rhythmicity and phase of arrest of host plant clocks can affect *P. xylostella* feeding.

4.4.6.2 Effect of *A. thaliana* JA mutants

JA mutant *A. thaliana* may have had similar affects due to the loss of signals *P. xylostella* may use to inform feeding rhythms, lacking functional genes key in JA pathways, which is shown to be important for glucosinolate and other herbivory deterrents' regulation (Brader, E and Palva, 2001, Halitschke and Baldwin et al., 2004, Kastell et al., 2013, Sánchez-Pujante et al., 2018). *aos* mutant *A. thaliana* lack a protein used in the initial steps of JA biosynthesis, which can also be produced in response to DAMPs (Damage-associated molecular patterns) demonstrating importance in plant responses to herbivorous attack (Weber, Vick and Farmer, 1997, Sivasankar, Sheldrick and Rothstein, 2000, Laudert and Weiler, 2002). *P. xylostella* larval feeding on *aos* mutants showed significantly more feeding in dark phase in L/D conditions and no significant differences between phases in L/L. Anticipatory feeding behaviours were also not maintained compared to WT L/D *A. thaliana*. Both L/D and L/L conditions were shown however to have significant feeding rhythmicity, showing (s) dark phase centred phase peaks and a significantly reduced rhythmic amplitude in L/L compared to L/D, likely showing why no significant differences were found between total feeding across L/L phases. The loss of rhythmic features compared to WT *A. thaliana* feeding rhythms is similar to clock mutants and OP *B. rapa* feeding. Other tested JA mutants show similar results, lacking certain rhythmic features. *opr3*, which affects a step further down the JA biosynthesis pathway (Schaller et al., 2000), with a potential OPR3 independent pathway that may allow for continued JA related biosynthesis pathways (Chini et al., 2018, Wasternack and Hause, 2018), showed non-significantly different phase peak to WT L/D. However, feeding between (s) midday-midnight is not significantly different from (s) light phase, suggesting that there is no notable anticipatory feeding around such, with significantly altered rhythmic amplitude and phase peak in L/L compared to WT. *coi1-16*, a mutant that blocks JA signalling, allowing for the completion of JA biosynthesis pathways and possible COI1 independent

signalling (Xie et al., 1998, Blechert et al., 1997, Stintzi and Browse, 2000), showed opposite condition significant differences to *opr3* when compared against WT. Both *opr3* and *coi1-16* mutant feeding totals were significantly lower than both WT *A. thaliana* conditions and *aos* L/D. The difference in mutants at varying steps of JA pathways may have slight effects on *P. xylostella* feeding, however, in general *P. xylostella* feeding across these mutants is reduced, with loss of anticipatory behaviours and rhythmicity that was found maintained into L/L conditions. *opr3* showed a somewhat increased anticipation slope though was not significant, yet, *opr3* was also the only mutant *A. thaliana* in L/D conditions with a non-signficantly different phase peak compared to WT. *opr3* independent JA biosynthesis can occur, therefore further host plant defence signals may have been present (Chini et al., 2018, Wasternack and Hause, 2018). The change in *A. thaliana* leaf nutrition and rhythmic quantities of herbivory deterrents across the mutants used may have caused the recorded changes through similar effects discussed for OP conditions and clock mutants. *P. xylostella* artificial diet can require brassica plant-based extracts to stimulate feeding (Carpenter and Bloem, 2002, Collins et al., 2010). Though the tested JA mutant *P. xylostella* may lack herbivory deterrents that otherwise restrict levels of feeding, without such attraction signals, likely from glucosinolate, *P. xylostella* is less attracted to leaf tissues (Yan et al., 2014, Li et al., 2018, Badenes-Perez, Gershenzon and Heckel, 2020), limiting any recorded increases in feeding from occurring. Many other species can use glucosinolates as a feeding attraction signal from host plants (Wittstock et al., 2003), therefore loss of such may reduce feeding as seen in our JA mutant results. The loss of rhythms in signals from JA regulated pathways, including general arrythmia caused by clock mutants, may also restrict or dampen the typical *P. xylostella* feeding rhythms previously recorded on IP *B. rapa* and WT *A. thaliana*.

4.4.6.3 *A. thaliana* clock mutant choice assay

When given the choice between feeding on WT or clock mutant *A. thaliana*, *P. xylostella* larvae showed no significant differences between WT and TM, including when both *cca1-ox* and TM were offered. However, in addition to the significantly reduced total feeding on *cca1-ox* shown in Fig. 4.6 and Table 4.8, Fig. 4.7 shows how across time points ~80% of feeding occurred on WT *A. thaliana* compared to *cca1-ox*, this shows *P. xylostella* larvae had a significant preference for WT leaves. This is different from previous findings on a generalist insect herbivore (*T. ni*) which showed significantly more leaf areas loss from *cca1-ox* compared to WT *A. thaliana* (Goodspeed et al., 2013a), though other species have shown reductions also (Lei et al., 2019). As discussed previously (in 4.4.6.1), features of *cca1-ox* leaves are potentially unattractive and my restrict feeding, including by *P. xylostella* larvae, which may have been otherwise attracted by the possibly increased glucosinolate presence. The loss of circadian clock regulation in TM *A. thaliana* doesn't appear to significantly affect feeding preference compared to WT, demonstrating that the potential release of host plant volatiles lacking rhythmicity does not affect host plant choice at the times sampled. However, when paired with *cca1-ox*, though this mutant shows significantly lower preference compared to WT, *P. xylostella* larvae do not prefer TM over such, demonstrating that the loss of circadian clock regulation in TM affected the preference for *cca1-ox* leaves by *P. xylostella* larvae. As previously discussed for *cca1-ox*, this may be due to the lower nutrition that may be found in TM leaves due to a loss in circadian clock facilitated plant fitness.

4.4.7 Conclusion

Throughout the results in this chapter, interactions between both *P. xylostella* and host plant signals are evident, demonstrating *P. xylostella* larval diel feeding rhythms that are controlled by both environmental signals and endogenous circadian clock regulation.

Previous research has shown that host plant rhythms and volatiles can have significant effects on not only the regulation of pest species behaviours (Goodspeed et al., 2013b, Fenske et al., 2018) but also their genetics (Grayson et al., 2015, Wybouw et al., 2015). Studies on *P. xylostella* specifically have shown circadian rhythms and responses to host plant volatiles that affect genetic expression and the regulation of behaviours such as mating and oviposition (Hillyer and Thorsteinson, 1969, Pivnick et al., 1990, Campos, Schoereder and Sperber, 2004). This, in combination with identified attraction responses to glucosinolates specific to *P. xylostella* preferred host plants (Feeny, 1977, Reddy and Guerrero, 2001, Yan et al., 2014, Li et al., 2018, Badenes-Perez, Gershenzon and Heckel, 2020), demonstrates the possibility for significant interactions between the *P. xylostella* and host plant defining herbivory. Larval *P. xylostella* exhibited defined feeding rhythms on *B. rapa* IP plant leaves, showing a distinct dawn trough and dusk peak in feeding that was maintained into L/L. This rhythm matches both features from previously researched nocturnal lepidopterans and *P. xylostella* activity rhythms, along with avoidance of host plant defences and potential artificial control measures. This precise rhythm was found to be reliant on the host plant clock, as when *B. rapa* leaves were OP with *P. xylostella* larvae significant rhythmic changes occurred, loosing anticipatory feeding activity and maintaining (s) dark phase centred feeding. This shows larval *P. xylostella* rhythms may also be maintained endogenously, with qPCR of clock gene transcripts showing consistent relative expression rhythms of core clock genes *per* and *tim* in both L/D-L/L and IP-OP conditions. The expression of GSS genes was also somewhat maintained into constant conditions and on OP *B. rapa* host plants, demonstrating that the regulation of GSSs, crucial to *P. xylostella* ability to feed on brassica crops, was maintained endogenously, though could be affected by host plant circadian clock phase such as with *Sulf3* relative expression. The specific plant host may be a determining factor of feeding rhythms also, with minor changes in rhythmic features found on WT *A. thaliana* hosts, however, *B. rapa*

feeding rhythms were largely maintained on *A. thaliana* without multi-generational prior entrainment. Moreover, the loss of host plant clock and JA gene functions led to feeding rhythms similar to *B. rapa* OP feeding, potentially demonstrating a typical nocturnal feeding rhythm that *P. xylostella* may maintain on varying hosts. These findings also showed that both plant clock function and the phase of such can determine *P. xylostella* larval feeding, with JA correlated defences important for signalling such. In addition, *P. xylostella* larval feeding on JA mutants with possible redundant JA defence pathways still active potentially show partial maintenance of WT feeding rhythmic features. These findings highlight the need for species-specific rhythm analysis between pests and hosts. The recorded rhythmic data, along with total feeding levels, may help inform how specialised insect herbivores, in addition to *P. xylostella* specifically, may interact with host plants and their circadian rhythms. These findings lay the groundwork of precisely timing control measures to interact with *P. xylostella* larvae when they are most exposed and show down-regulation of detoxification genes, as well as understanding how the regulation of host plant circadian rhythms and defences may impact pest herbivory and circadian rhythms.

Chapter 5 Discussion, Conclusions and Future Directions

5.1 General discussion

5.1.1 Light and abiotic responses

Temperature is well known to affect the general activity of organisms, especially ectotherms (Schulte, 2015), with increasing temperatures often eliciting increased rates of activity up to a peak threshold (Schou et al., 2013, Sinclair et al., 2016). Lepidoptera are largely ectothermic along with most Arthropoda, however lepidopterans can generate their

own body heat through 'shivering' prior to flight, specifically targeting flight muscles (Kammer, 1965, Heinrich and Mommsen, 1985, Heinrich, 2007). Therefore, it is likely *Plutella xylostella* may interact with temperature changes, however, changes in activity may not be temperature-dependent, with our results showing that both increases and decreases in temperature elicited increased activity in adults. Temperature change over time can also inform seasonal changes which may be used by lepidopterans for triggering migratory behaviours, as shown in *Danaus plexippus*, a widely researched migratory lepidopteran, with migration triggered via cooling temperatures and maintained using circadian clock assisted compass mechanisms (Froy et al., 2003, Reppert and Roode, 2018). As *P. xylostella* show seasonal migrations in the wild, temperature may be a migration signalling factor, however in contrast to *D. plexippus*, *P. xylostella* shows little evidence for return migrations during cooling temperatures (Wei et al., 2013), with it being currently unclear how *P. xylostella* migration interacts with circadian clocks. In addition, a recent paper showed temperature may also interact to elicit circadian activity rhythms in D/D (Constant dark) which otherwise is not maintained by *P. xylostella* adults (Wang, Yang and Chen, 2021), with no maintenance also seen in our results. In this aforementioned paper, *P. xylostella* reared at 26°C produced similar arrhythmic results to our own experiments, but when the temperature was dropped to 20°C adult male *P. xylostella* maintained rhythmic locomotor activity in D/D. We specifically used 20°C to test whether this temperature allowed for maintenance of circadian rhythms with our strain of *P. xylostella* in D/D following a 12/12 L/D (Light/Dark) cycle, in between the 14/10 and 10/14 cycles used in the previous paper, and found no maintenance of rhythmic locomotor activity. Further research on the affect of temperature on eliciting *P. xylostella* behaviours is needed, however, in general our results showed that temperature has a smaller influence on *P. xylostella* activity than changes in light, with light often being a more dominant zeitgeber (Kaniewska et al., 2020), which we show also affects *P. xylostella* larvae

significantly. This is seen in many other species, including *D. melanogaster* (Rensing and Ruoff, 2002, Yoshii et al., 2009, Refinetti, 2010, Kannan et al., 2012). There was increased larval feeding leading up to (s) dark phase onset in in-phase (IP) conditions on *Brassica rapa* and *Arabidopsis thaliana*. In the wild, insect activity peaks have been shown during the evening possibly due to the reduced light levels reducing predation (Clarke, 1983, Thomas and Jacobs, 2013, Kirkeby, Wellenreuther and Brydegaard, 2016) while maintenance of higher temperatures allow for increased insect activity (Mellanby and Gardiner, 1939, Schou et al., 2013, Sinclair et al., 2016), therefore endogenous circadian rhythms that can exploit this natural cycle may be present in *P. xylostella* larval feeding, leading to our results. However, other abiotic factors such as relative humidity may interact with *P. xylostella* behavioural rhythms also. Humidity has been shown to significantly affect *P. xylostella* life stages, specifically showing increased humidity causing negative effects to larvae, in addition to rainfall and irrigation (Lim, 1982, Lee and Huang, 1986, Guo and Qin et al., 2010, Machekano et al., 2019, Farias et al., 2020). Our results showed that *P. xylostella* larval feeding rhythms had troughs around morning on IP *B. rapa* plants which may be in time with peaks in humidity and irrigation schedules (Mwimba et al., 2018, National Weather Service, 2022), leading to an adaptive benefit for reduced activity during this time, however, it is unclear how such may interact with *P. xylostella* circadian clocks and entrainment.

One of the most ancient and simplest clock functions is the timing of division in anticipation of sunrise in cyanobacteria via the sensing of redox state in photosynthetic pathways (Cohen and Golden, 2015, Diamond et al., 2015). Changes in light conditions are responsible for these changes as well as being the most predominant zeitgeber used by organisms, as the planet rotates every ~24 hours (h) (Pittendrigh, 1993, Schmal, Herzel and Myung, 2020). Light repression of activity is widely seen across life, with large numbers of organisms opting for nocturnally active behaviour (Clark, 1914), which is used to

provide specific survival benefits (Daily and Ehrlich, 1996). Moths themselves, as part of Lepidoptera, diverge from butterflies temporally via the selection of opposing diel activity rhythms (Kawahara and Breinholt, 2014, Kawahara et al., 2018), with our adult *P. xylostella* results and some larval findings matching this common nocturnality. Avoiding activity during light phases, like we show for *P. xylostella*, is common due to increased risks of predation under light exposure (Longcore and Rich, 2004), however moths can have both their activity repressed by light but also can be attracted to it such as with ALAN (Artificial light at night) (Macgregor et al., 2014). Human light sources can disrupt many key physiological processes in moths from feeding (Langevelde et al., 2017) and reproduction (Nemec, 1969, Sower et al., 1970) to pollination (Macgregor et al., 2014) and detection of predators (Svensson and Rydell 1998). *P. xylostella* may preferentially avoid activity in the morning, shown in both adult and larvae activity, as predators may use morning light to hunt lingering nocturnal prey with previous research showing higher predator foraging efficiency around this time (Kerckhove, Shutter and Abrams, 2012). Circadian timing/anticipation to avoid predators is shown across ecological settings (Kohl et al., 2018), and may too be a driving factor influencing lepidopteran behaviours (Daily and Ehrlich, 1996, Gordan, Dickman and Thompson, 2010) and therefore the conserved trough in activity we find across both adult and larval *P. xylostella* activity. With reduced light in evening periods also potentially allowing for increased activity due to lower pressure from diurnal predators (Clarke, 1983, Thomas and Jacobs, 2013, Kirkeby, Wellenreuther and Brydegaard, 2016), allowing for the adaptation of endogenous rhythms of activity that increase near dark phase onset as we find for IP *P. xylostella* feeding. There are many examples of L/L(Constant light) having strong repressive effects on activity and behavioural rhythms, matching our findings for adult *P. xylostella*, with the lepidopterans *D. plexippus*, *Spodoptera littoralis*, *Plodia interpunctella* and *Ephestia kuehniella* all showing a loss of activity rhythms under L/L conditions (Froy et al., 2003, Syrova,

Sauman and Giebbultowicz, 2003, Zavodska et al., 2012), somewhat similar to the larval *P. xylostella* findings also reported in this paper. Shading of micro-habitats from both abiotic and biotic factors provided by host-plant foliage (Landsman and Thiel, 2021) may reduce the aversive responses to light found in *P. xylostella* larvae compared to adults, which in contrast show immediate highly significant reductions in activity in response to such, also becoming arrhythmic.

5.1.2 Circadian clock molecular rhythms

Clock genes are conserved across most animals, including similar models of function through TTFL (Transcription and translation feedback loops) being found across life in general. *P. xylostella per* (*period*) and *tim* (*timeless*) core clock component genes were found and through qPCR we showed their cyclic expression rhythms are similar to other lepidopteran findings (Zhu et al., 2008, Merlin, Gegear and Reppert, 2009, Kobelkova et al., 2015, Yan et al., 2017, Zhang et al., 2021). Our results also showed the same relative expression rhythms between both adult and larval *P. xylostella,* demonstrating a conserved mechanism of circadian clock function after complete metamorphosis, additionally showing the same rhythms in constant conditions and from OP (out-of-phase) fed larvae. This demonstrates, in spite of adult *P. xylostella* not showing strong circadian rhythms in locomotor behaviour, that *P. xylostella* circadian clock function is both present and robust, including compared to some other lepidopterans (Merlin, Gegear and Reppert, 2009, Kobelkova et al., 2015, Zhang et al., 2021). Other clock genes are likely important in the regulation of observed circadian rhythms and responses. *Pyrrhocoris apterus* (European firebug) that lacks *Drosophila*-like (d)CRY (Cryptochrome) shows maintenance of activity into L/L, potentially due to the lack of the direct light-sensitive input into the core molecular circadian clock (Kaniewska et al., 2020). The effect dCRY has on TIM in *P. xylostella* may be investigated both pre- and post-metamorphosis in relation to the different

response to L/L in adults compared to larvae. However, rhythmic locomotor activity is lost in both D/D and L/L in adult *P. xylostella*. The expression of *cry* genes is often limiting for low-light sensitivity and its photoresponsiveness in activity and stability leads to the rundown of the molecular circadian clock (Emery et al., 1998). Adult *P. xylostella* show high sensitivity to light and their *cry* activity may be important for circadian and other physiological functions. In other members of Lepidoptera, *cry* has been shown to be crucial in migration and other circadian regulated responses (Zhang et al., 2017, Wan et al., 2021), interacting with *per* and *tim*, regulating their expression (Yuan et al., 2007, Kyriacou, 2009). As a migratory pest, understanding how *cry* is expressed may therefore be important. *per* and *tim,* as part of the core molecular clock, have also been shown to be expressed in a variety of tissues among a number of lepidopteran species, however most research has been carried out in *P. xylostella* on immune and feeding associated genes (You et al., 2013, Xia et al., 2015, Yu et al., 2015, Ma et al., 2018). *P. xylostella* larvae showing relative expression rhythms in clock genes from whole body samples demonstrates circadian clock regulation. However, it is unclear whether peripheral clocks are found in the midgut. If so, midgut and possibly other digestion-related peripheral clocks can be examined for synchrony with CCN (Central clock neurons) in the presence of varying zeitgebers (Sauman and Reppert, 1998, Giebultowicz, 2001, Stokkan et al., 2001, Nobata et al., 2012, Bajgar, Jindra and Dolezel, 2013). Our *per* and *tim* expression results may represent various tissues within the whole *P. xylostella* larval body. Divergent phase relationship among different clocks, for example in response to OP feeding, could be masked if one of these clocks is dominant in it represented expression levels. Robust clock gene expression has also been reported for *D. plexippus* adult head samples, another multi-tissue structure (Zhu et al., 2008). Multi-tissue preps may still offer a convenient means of detecting rhythmicity across a variety of output genes. For example, the expression of

detoxification genes involved in *P. xylostella* resistance pathways may be worth examining in whole larvae.

5.1.3 Regulation of sulfatase genes

Detoxification genes such as GSSs are unlikely be expressed in CCN, with Ma et al. 2018 showing that *Sulf2* and *Sulf3* are expressed in multiple regions of the *P. xylostella* larval body, mostly excluding the head (Ma et al., 2018). Whole-body samples were, therefore, used to examine GSS expression in response to test conditions. Sulfatase genes are harnessed by many species to detoxify plant secondary metabolites and show diversity across insect herbivores (Beran et al., 2018, Manivannan et al., 2021, Ren et al., 2021). In addition to *P. xylostella*, another brassica feeding specialist the Coleopteran species *Phyllotreta armoraciae*, appears to have overcome the MOB (Mustard oil bomb) instead by targeting myrosinase for inactivation and rapidly absorbing glucosinolates while also showing minimal effects from the toxic hydrolysis products (Sporer et al., 2021). Another species of Coleoptera, *Psylliodes chrysocephala*, seems to have independently evolved a family of unique GSSs (Glucosinolate sulfatase) that specifically target benzenic glucosinolates (Ahn et al., 2019). *Bemisia tabaci* (Silverleaf whitefly) has been identified as another brassica herbivore with GSS. As a phloem feeder, it has also become attracted to the indolic glucosinolates which its GSS family is specialised to desulphate (Manivannan et al., 2021) which is also upregulated in response to phloem feeder attack (Kim and Jander, 2007, Widemann et al., 2021). As *P. xylostella* appears to have GSS proteins with universal glucosinolate interactions, this may be why *P. xylostella* is attracted to the overall profile of glucosinolates (Robin et al., 2017, Heidel-Fischer et al., 2019) instead of subsets like shown in *B. tabaci*. The sulfatase genes we tested were shown to likely be the most important for MOB disarmament (Ma et al. 2018, Heidel-Fischer et al., 2019) and showed notable rhythmic expression maintained into varying conditions,

indicating endogenous circadian regulation. The combined primer *Sulf2+3*'s *(Plutella xylostella sulfatase 2* and *3)*, used to bind to and amplify both *Sulf2* and *Sulf3* transcripts in case the singular specific primers were ineffective due to close sequence similarity (Ma et al., 2018), expression profile matched both *Sulf2* and *Sulf3* in L/D conditions, then matching only *Sulf2* in L/L. This last finding may indicate that *Sulf2* is the more predominant transcript, similarly shown by Ma et al., and further shows GSS genes possible interactions with L/D cycles. GSS genes would likely also need to be expressed in anticipation of glucosinolate presence due to GSS proteins having to competitively inhibit the action of myrosinase (Ratzka et al., 2002, Singh, 2017) and so must respond to secondary metabolites and anticipate the accumulation of such. This possible need for anticipatory responses likely makes it beneficial for GSS expression to be under *P. xylostella* circadian clock regulation. Our results suggest that though GSS expression matches potential exposure to glucosinolate in brassicas, OP changes in such didn't cause immediate shifts in GSS regulation. Though larval *P. xylostella* feeding rhythms on mutant *A. thaliana* matched OP *B. rapa* feeding, it is unclear how the expression rhythms of GSSs changes. Heidel-Fischer et al., 2019 showed *Sulf2* and *Sulf3* transcripts were less abundant in *P. xylostella* larval midguts when reared on met-glucosinolate mutant *A. thaliana*. This showed that GSS genes are inducible by glucosinolate presence (Heidel-Fischer et al., 2019), however it's unclear over what time scale entrainment to or stimulation by occurs. Our results showed no *Sulf2* or *Sulf3* direct 12h shift in expression across time in OP *B. rapa* feeding. Glucosinolate and or other volatile signals from host plants may therefore entrain the diel expression of GSS genes by the *P. xylostella* circadian clock over time as *Sulf2* expression was 'in-time' with previously recorded glucosinolate presence from IP host plants (Goodspeed et al., 2013b) prior to experiment set-up. *Sulf3* on the other hand showed significant differences on OP *B. rapa* as well as there being changes in rhythmic features between L/D and L/L. This showing that L/D cycles may interact with the

rhythmic expression of genes, increasing rhythmic amplitude (Wijnen et al., 2006), and that *Sulf3* may be more responsive to changes to glucosinolate expression, most likely aliphatic glucosinolates which it interacts with and is proportionally found at higher levels in leaf tissues (Hopkins, Dam and Loon, 2009, Sonderby et al., 2010, Ishida et al., 2014, Heidel-Fischer et al., 2019).

5.1.4 Regulation and maintenance of diel rhythms

Circadian regulation of activity across physiological processes is widely recorded throughout many species (Pittendrigh, 1993, Foster and Kreitzman 2005, Numata, Miyazaki and Ikeno, 2015). In Lepidoptera, numerous studies have shown rhythmic activity in biological processes with evidence of circadian mechanisms controlling the precise timing of a number of lepidopteran behaviours (Brady et al., 2021). The circadian rhythms and location of circadian control has been studied in silk moths such as *Hyalophora cecropia*. The brain of *H. cecropia* contains CCN, being able to maintain eclosion and flight activity rhythms even after the loss of key sensory organs (Truman, 1972, Truman, 1974). This demonstrates true circadian rhythmicity as behavioural rhythms were maintained endogenously. Maintenance of endogenous rhythms across lepidopteran species is also evident in D/D conditions as shown in both *Hyles lineata* and *Manduca sexta* hawkmoths (Broadhead et al., 2017). Circadian control of activity rhythms are found at both behavioural and gene expression levels in both moths and butterflies however there can be noticeable species-specific differences between such (Merlin, Gegear and Reppert, 2009, Kobelkova et al., 2015, Niepoth et al., 2017). It is also unclear how individual species may react to light cycles and constant conditions with different species of moth having different activity peaks and reactions to D/D conditions. *E. kuehniella* is dawn active and can maintain activity rhythms in D/D while *P. interpunctella* is dusk active and arrhythmic in constant conditions (Zavodska et al., 2012). These findings demonstrate that

Lepidoptera, such as *P. xylostella*, likely show both rhythmic behaviours and express rhythmic molecular circadian clocks. Our results from both adults and larval *P. xylostella* show activity rhythms are present across many conditions, however, while larval data shows evidence of true circadian rhythmicity, adult *P. xylostella* appear to react directly to diel changes in environmental conditions. Adults show a release of 'pent up' activity at dark phase onset which leads to the lowering of dark phase activity at later dark phase h, rather than towards a light phase onset trough as recorded for *P. xylostella* larval feeding on IP *B. rapa* and on wild type (WT) Col-0 *A. thaliana* in L/D. Constant condition experiments on *D. melanogaster* over many generations where influences of non-light signals are minimised have demonstrated maintenance of behavioural rhythms, even increasing in robustness over time in D/D (Shindey et al., 2017). This and previously discussed examples (in 1.2.2), help to demonstrate the ubiquitousness of biological rhythms and, in addition to the highly conserved presence of circadian clocks across species, allude to the large adaptive benefit circadian rhythms offer life (Sharma, 2009). Therefore, the lack of circadian control of adult *P. xylostella* locomotor activity is unexpected and without circadian control of recorded behaviours, there are likely other regulatory mechanisms that control *P. xylostella* adult activity.

Other species have shown significantly reduced rhythms in constant conditions and the run down of clock gene rhythmic expression (Merlin, Gegear and Reppert, 2009, Kobelkova et al., 2015, Zhang et al., 2021). In addition to the rundown of rhythmic behaviours we recorded in *P. xylostella* larvae, adults showed immediate light repressive effects and almost no activity in L/L over time. Larval feeding in IP conditions also decreased to a certain extent under prolonged L/L. This aversion to light activity has been previously discussed (in: 3.4.2, 4.4.1), however, it has also been found that *P. xylostella* is the least attracted to blue light LEDs compared to other colours and tested crop pests (Cho and Lee, 2012, Park and Lee, 2017), further exemplifying a possible avoidance of daytime periods

rich in blue light (Knoop et al., 2019). By attempting to maintain activity responding directly to dark phase periods and presence of light as we show, helping avoid predation, *P. xylostella* may maximise fitness. The homeostatic modulation of activity may instead regulate the locomotor behaviour captured in our adult *P. xylostella* experiments. Sleep homeostasis is a widely researched regulation of behaviour that can be independent of circadian control, where the possible accumulation of 'sleep debt' leads to organisms attempting to maintain a certain level of sleep on average (Cirelli and Bushey, 2008, Deboer, 2018) and therefore total activity levels. Other studies have found compensation in the distribution of activity to maintain overall constant rates with *Manduca sexta* (Tobacco hornworm) larvae showing constant arrhythmic feeding behaviour that reduces gaps in-between feeding bouts to maintain constant growth rates on both plant and artificial diets (Reynolds, Yeomans and Timmins, 1986). *Lynx lynx*'s (Eurasian lynx) 24h activity levels also remained constant, unaffected by shifting photoperiods while being significantly less active during the day (Heurich et al., 2014), demonstrating similar homeostatic modulation of behaviour. Other limits and non-circadian modulations in behaviours have previously been recorded. *Locusta migratoria* (Migratory locust) show few limits in the amount eaten during a feeding phase but instead is limited by length of time of such (Simpson, 1982). To maintain adequate protein supply from nitrogen poor food sources *L. migratoria*, limited by length of feeding phase, instead reduced gaps between feeding bouts (Simpson and Abisgold, 1985), similar to *M. sexta*. Length of starvation periods is non-limiting at low and limiting at high lengths for *M. sexta* larvae also, as they can increase their meal size up to a certain extent to compensate (Timmins and Reynolds, 1992). Similar mechanisms may determine adult *P. xylostella* results, showing homeostatic control rather than circadian due to certain adaptions and traits. Our results show, similarly to *M. sexta* and *L. migratoria*, expression of further activity in light phase to compensate for reduced time available during dark phase, homeostatically maintaining a particular amount of activity. As

previously discussed (in: 3.4.2, 4.4.1), *P. xylostella* as a nocturnal lepidopteran may find it advantageous to avoid activity during light phases, e.g. to avoid predation, with feeding, mating and oviposition occurring during the dark phase where previous studies have shown *P. xylostella* to carry out most of these activities (Pivnick et al., 1990, Lee, Lee and Boo, 2005).

However, another key aspect of *P. xylostella* to consider is its extensive migrations. *P. xylostella* migrates seasonally, invading brassica crops grown at higher latitudes as temperatures warm, successfully invading regions as long as temperatures remain elevated due to a lack of an ability to diapause (Honda, 1992, Campos, Schoereder and DeSouza, 2006, Furlong, Wright and Dosdall, 2012). Sensitivity to temperature, as discussed previously (in: 1.3.1.3, 3.4.6, 5.1.1), may allow such to act as a migration cue due to its strong effect on the *P. xylostella* life cycles, rather than photoperiod as seen in other animals (Sockman and Hurlbert, 2020). Other insect migrations likely rely on photoperiodism, but it is unclear how much such interacts with circadian mechanisms, with *D. plexippus* showing migratory responses to temperature change, though harnesses circadian clocks during migration also (Froy et al., 2003, Bradshaw and Holzapfel, 2010, Reppert and Roode, 2018). Insensitivity to photoperiodic entrainments may be advantageous to *P. xylostella*, opting for direct response to light conditions as we show, due to the large changes in light conditions associated with the scale and speed of *P. xylostella* migration (Talekar and Shelton, 1993, Coulson et al., 2002). 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 (Campos, Schoereder and Sperber, 2004). Further testing also showed that photoperiod had no discernible effect on *P. xylostella* morphological and life history traits (Campos, 2008). As adult *P. xylostella* appears to show minimal endogenous interactions to specific photoperiods, similar results can therefore be expected for light

schedule profiles as shown in our results, relying on alternative control mechanisms, i.e. homeostatic regulation. Further evidence from adult *P. xylostella* results of no circadian control, comparatively to larval finding, is the lack of anticipatory behaviour. This is another difference to Wang, Yang and Chen's 2021 paper which showed a certain amount of dark phase anticipation. Phase anticipation is a sign of active circadian control of the behaviour (Stoleru et al., 2004), however, throughout all our recorded results there are no notable increase in light phase activity in adult *P. xylostella* prior to dark phase onset. Previous papers have shown *per* to be required for anticipatory behaviours (Grima et al., 2004), which our results show *P. xylostella* adults have for the former but lack the latter, however, other findings have shown that *per* expression may not be indicative of circadian behaviours as they can persist using *Clk* (*Clock*) and *cyc* (*cycle*) expression (Goda et al., 2011). Without anticipation, our results show a predominant dark phase onset peak in activity, increasing in proportion of total activity as light phase periods are extended in L/D cycles, potentially showing a release of 'pent up' activity. Similar results are seen from starvation period experiments on *M. sexta* larvae, increasing their meal size by ~3x following increased foodless period lengths (Timmins and Reynolds, 1992). These extended light phase light cycles may also trigger bimodal activity (Zavodska et al., 2012), however *P. xylostella* adults show no evidence of this, continuing to react directly to presence of light with modulation of total activity and distribution between phases. These adult *P. xylostella* findings and feeding data from larvae (which do show anticipation and evidence for circadian clock regulation) may be induced by responses to host plant volatiles, with previous research indicating that interactions with such can cause significant changes to both *P. xylostella* behaviours and physiology (Pivnick et al., 1990, Carpenter and Bloem, 2002, Lohr and Kahuthia-Gathu, 2002, Campos, 2008, Collins et al., 2010, Zhu et al., 2016).

5.1.5 Role of plant and other biotic signals

Both sex and plant pheromones can have many effects on organisms including lepidopterans and they have been found to interact with each other to elicit lepidopteran sexual behaviours. *Helicoverpa zea* (Corn earworm) responded to presence of sex pheromones in experiments by increasing activity levels and increased flight tendencies (Crespo, Goller and Vickers, 2012), this may be similar to the results seen in our group assay with adult *P. xylostella* where D/D conditions had significantly more total activity compared to 12/12 L/D, in contrast to standard locomotor assays where moths were isolated. Conspecifics have been shown to signal social entrainment in eusocial insects (Mistlberger and Skene, 2004, Fuchikawa et al., 2016), additionally interactions with other species as food sources and predators can also act as zeitgebers (Prugh and Golden, 2014, Bennie et al., 2018, Kohl et al., 2018), demonstrating how species interactions may influence the expression of circadian rhythms.

Interactions with plants have been shown to have significant effects on both insect behaviour and community composition, often through plant-pollinator networks (Bloch et al., 2017, Fenske et al., 2018, Burkle and Runyon, 2019). Host plant signals also likely significantly interact with pest species. *Spodoptera frugiperda* (Fall armyworm) and *Chloridea virescens* (Tobacco budworm) larvae fed diet containing plant material displayed differences in circadian rhythms of feeding compared to those fed on non-plant extract containing artificial diet, with similar results seen in adults fed on diet containing plant material, having altered activity and flight and light responses (Miranda-Anaya, Guevara-Fefer and Garcia-Rivera, 2002). This shows that the provision of host plant volatiles, even when arrhythmic, can have significant effects on the rhythmic behaviours of species. This including *P. xylostella*, showing significantly altered sexual activity in response to host plant volatiles (Pivnick et al., 1990), making it clear there is potential for our results to be significantly influenced by plant signals. Studies have repeatedly indicated

a close relationship between the circadian rhythms of Lepidoptera and host plants (Hoballah et al., 2005, Goodspeed et al., 2012). Adult *M. sexta* feed rhythmically on plant nectar and show interactions between circadian rhythms of adult and host plant volatile signals. This rhythmic relationship has been shown to be crucial to the success of pollination services on *M. sexta*'s host plants. When the rhythmic interactions are altered via OP rearing between *M. sexta* and *Petunia axillaris* (White petunia) host plants, the loss in pollination efficiencies is evident due to significantly reduced flower visits (Fenske et al., 2018). This loss of efficiencies and benefit to plant hosts is also evident in larval herbivory. Studies have shown that in both the brassicas *Brassica oleracea* and *A. thaliana*, the accumulation of glucosinolates in plant tissues is rhythmic, peaking during the day and found at the lowest levels during the night (Goodspeed et al., 2013b). These rhythms in secondary metabolite content have been shown to provide a certain level of resistance to herbivory, such as reducing the amount of tissue loss under generalist lepidopteran herbivore (*Trichoplusia ni*) attack when compared to arrhythmic *A. thaliana* mutants (Dodd et al., 2005, Goodspeed et al., 2012, Goodspeed et al., 2013b). The feeding rhythms of *T. ni* have been shown to both be rhythmic and peak in feeding approximately around evening on artificial diets. *T. ni*'s weight gain increased significantly in both L/L and D/D conditions equally when fed on *B. oleracea* compared to L/D cycles (Goodspeed et al., 2013b). More notably however, is that when fed on host plants OP to *T. ni*, *T. ni* showed significantly greater weight gain by nearly 4x than IP host plants (Goodspeed et al., 2012), with similar findings reproduced across a selection of non-brassica crops. However, by 6 days of feeding on OP *B. oleracea,* there was no longer any significant difference between IP and OP fed *T. ni* weight gain (Goodspeed et al., 2013b). Our results showed similar interactions between pest and host plant circadian rhythms, however with differences potentially related to either *P. xylostella* endogenous physiological rhythms and or brassica feeding specialism. Compared to *T. ni* OP feeding results, *P. xylostella* showed minimal

changes in total feeding with no significant increases compared to IP with similar shown for larval weight gain. This may be due to the maintenance of entrainment to IP *B. rapa* the *P. xylostella* has been reared on, also potentially leading to the slight differences when exposed to *A. thaliana* in L/D, with prior entrainment to such only occurring within a *P. xylostella* generation. However, *T. ni* also showed a potentially delayed entrainment to changes in plant signals, losing the significant increase in weight gain after 6 days exposed to OP plant tissues (Goodspeed et al., 2013b). *P. xylostella* may require similar or longer lengths of time for feeding rhythms to entrain to shifts in host plant zeitgebers, however, this finding would also show that plant volatile zeitgebers have a stronger effect on *P. xylostella* compared to light. Loss of aliphatic glucosinolate production via *myb28myb29* mutant *A. thaliana* leaf tissues also showed significantly altered feeding from *T. ni*, showing significantly increased leaf area loss and the same level of feeding that occured on OP leaves (Goodspeed et al., 2013b), similar to our results that showed feeding rhythms between OP *B. rapa* plants and IP *A. thaliana* mutants were similarly disrupted. This indicates that the maintenance of IP *P. xylostella* rhythmic feeding profiles is likely reliant on the presence of glucosinolates in host plants, or JA associated signals, with *myb28myb29* mutants likely having similar effect on *P. xylostella* feeding to the JA mutans we tested. Increases in *P. xylostella* OP feeding may have been restricted due to interactions between endogenous feeding rhythms being nocturnal, overlapping with shifted peak glucosinolate accumulation, in addition to overall GSS gene regulation not changing to match this shift shown in our qPCR results.

P. xylostella has been reared in the lab to select for *Pisum sativum* (Sugar snap pea) (Lohr and Gathu, 2011), recreating the jump in host plants seen in the wild in Kenya (Knolhoff and Heckel, 2011, Henniges-Janssen et al., 2011a,b). Achieving ~50% survival of *P. xylostella* on this new host plant in the lab took 4 generations of selection (Lohr and Gathu, 2011), showing *P. xylostella*'s adaptability but also that entrainment to new host plants

likely takes more than 1 generation, such as the entrainment to *A. thaliana* rearing conditions used for experiments. Changes in plant volatiles caused by the mutant *A. thaliana* used therefore also may take time for *P. xylostella* to adapt to, similar to OP *B. rapa* results. However, the loss of rhythms in plant circadian clocks or secondary metabolite expression may lead to absence of rhythmic entrainment. The loss of these rhythms and glucosinolate signals may have led to the somewhat decreased total feeding levels we also found, as it has been shown that *P. xylostella* relies on such for host-plant attraction (Yan et al., 2014, Li et al., 2018, Badenes-Perez, Gershenzon and Heckel, 2020). The more notable significant reduction in total feeding on *cca1-ox* (*Circadian clock associated 1– overexpressor*) *A. thaliana* may have occurred from the up-regulation of herbivory defence pathways (Lei et al., 2019, Zhang et al., 2019, Yamaura et al., 2020), leading to increases in myrosinase *P. xylostella* GSS genes may have not outcompeted as efficiently, in addition to increased defensive phenotypes such as increased trichomes (Green et al., 2002, Traw and Bergelson, 2003, An et al., 2011). The loss of leaf nutritional quality from these phenotypes and loss of rhythms (Green et al., 2002, Dodd et al., 2005) may also affect *P. xylostella* feeding as nutritional content and age of host plants have been shown to act as migration cues, with older host plants producing *P. xylostella* adults with favourable migratory traits (Campos, Schoereder and Sperber, 2004). These feeding results are also similar to findings from the phloem feeding pest *Myzus persicae* (Green peach aphid), showing decreased feeding in response to increased indolic glucosinolate levels in *cca1-ox A. thaliana* (Kim and Jander 2007, Lei et al., 2019). As *P. xylostella* is susceptible to the toxic products of such after MOB reactions (Li et al., 2000), these increased glucosinolate levels may have also inhibited larval feeding. As *Sulf2* appears to not immediately change expression on OP *B. rapa* in response to 12h phase shift in glucosinolate expression, with *Sulf3* action not interacting with indolic glucosinolates (Heidel-Fischer et al., 2019), this lack of response may lead to the found significantly

lowered *P. xylostella* feeding on *cca1-ox A. thaliana*. In contrast a generalist pest that doesn't rely on these specialised GSS genes (*T. ni*) however showed increased feeding on *cca1-ox A. thaliana* (Goodspeed et al., 2012), possible demonstrating that generalist pests, compared to *P. xylostella* and *M. persicae*, may be more able to adapt and outcompete shifts in host plant defence regulation, likely due to a lack of specialisation to specific host plants.

Further to the differences discussed between *B. rapa* and *A. thaliana* feeding rhythms and with Wang et al. 2021's adult *P. xylostella* paper, these differences may be the result of *P. xylostella* strain, rearing and locomotor set up. The *P. xylostella* strain used in this paper was collected from Rothamsted in the UK during the 1960's and has since been maintained on *B. rapa* plant cultures. Geneva 88, the strain used in Wang et al. 2021, has been maintained in lab cultures for a shorter period but mass reared on artificial diet. The increased time under lab culture conditions for the Rothamsted strain used here and presence of plant volatiles and subsequent interaction with plant circadian rhythms may have affected the expression of circadian behaviours in the *P. xylostella* strain used. With long-term effects of host plant entrainment shown on *Tetranychus urticae* (Red spider mite) (Wybouw et al., 2015). In locomotor assays specifically, the interaction of *P. xylostella* colonies with *B. rapa* before assay set up (Campos, Schoereder and Sperber, 2004) or the presence of volatiles from honey (Manyi-Loh, Ndip and Clarke, 2011), used by Wang et al, 2021, may also have affected the recorded data.

5.1.6 Applications to IPM

These findings show evidence of specific regulatory pathways and zeitgebers that may define *P. xylostella* circadian rhythms and interactions with both natural plant defences and IPM (Integrated pest management) strategies. Temporal considerations of pest management were first shown to be important in *Blattella germanica*, where these

cockroaches demonstrated varying susceptibility to pesticides over 24h (Beck, 1963), with recent research still uncovering the mechanisms behind rhythmic changes in resistance levels in *B. germanica* (Lin et al., 2014). It is unclear how many differing factors of an organism's rhythmic physiology and behaviour may change treatment susceptibility, as individual treatments, biotic and abiotic conditions can interact to have varying impacts on toxicity (Pszczolkowski, 2008). Therefore, it is important to consider the endogenous circadian rhythms of pest species before establishing treatment susceptibility, as the factors behind pest species treatment detoxification importance may be misinterpreted due to the interplay of the numerous interacting variables (Halberg et al., 1974). The use of species-specific activity rhythms, from both behavioural and gene expression data, which we now provide for *P. xylostella*, may be crucial for complimenting IPM strategies and the efficient use of control measures in addition to advancing our understanding of economically impactful pests. It has been previously shown that the use of control treatments on pest species are subject to effects of chronotoxicity (Hamby et al., 2013, Khyati, Malik and Seth, 2017), with *Spodoptera litura* (Tobacco cutworm) showing significantly increased mortality when treated with insecticide at certain times of day. These changes in levels of resistance can be closely tied to circadian rhythms (Shirasu-Hiza et al., 2007, Zhang et al., 2021). In addition to time-dependent effects of pesticides, IPM treatments may be improved by the harnessing of *P. xylostella* rhythms. *P. xylostella* bait traps have certain levels of effectiveness across the time of day (Furlong et al., 1995) as well as seasonally (Li et al., 2021). Specific trapping often relies on sex pheromones which have been shown to interact with the presence of plant volatiles (Pivnick et al., 1990, Li, Zhu and Qin, 2012). The inclusion of *P. xylostella* rhythmicity data we've found and how such rhythms may change seasonally in response to certain abiotic and biotic factors may be used to compliment such pest control treatments. SIT (Sterile insect technique) relies on the release of self-limiting strains of a pest to breed with WT populations such as that

developed for *P. xylostella* (Bolton et al., 2019, Shelton et al., 2020). The effective timing of release may be reliant on how activity may be expressed and subsequent interactions with natural conditions and plant volatiles, which we have shown to have significant effects on *P. xylostella* activity in addition to previous papers (Pivnick et al., 1990, Miranda-Anaya, Guevara-Fefer and Garcia-Rivera, 2002). Therefore, the understanding of adult activity rhythms and what controls such can be a crucial step to controlling *P. xylostella* pest populations, reducing damage to crops via the reduction of pest populations directly or through reproductive disruption by complimenting certain IPM strategies. Feeding rhythms of *P. xylostella* larvae are useful for pesticides and IPM strategies also, informing when *P. xylostella* may be most exposed to treatments (Shipp and Otton et al., 1976, Lin et al., 2014, Zhang et al., 2021). Our data also shows evidence of regulatory pathways controlling and interacting with these feeding rhythms as well as influencing total herbivory. Exposure to pesticides, parasitoids and other IPM strategies are increased when *P. xylostella* is most active, therefore matching treatments with our findings will increase their efficacy and therefore reduce both costs and environmental damage due to non-specific effects. Though weight gain data shows somewhat similar results, these do not directly relate to such exposures. The expression rhythms of GSS genes may also relate to general detoxification pathways which it has been shown *P. xylostella* can co-opt for pesticide resistance (Tao et al., 2012, Heidel-Fischer et al., 2019), therefore knowing when they are upregulated may be important for improving efficacy through considerations of chronotoxicity. Shifting expression of natural plant defences in host plants may also lead to increased herbivory resistance due to the limited increase in feeding and shifts of GSS expression in OP and mutant host plant conditions we found, however, it remains unclear what specific interactions entrains these rhythms in *P. xylostella* and whether limiting *P. xylostella* feeding via these methods is sustainable long-term due to *P. xylostella*'s adaptability.

5.2 Conclusions

The research and findings in this thesis have led to the following conclusions:

1. The literature shows that the application of circadian rhythms to IPM has the potential to increase treatment efficacy by the precise timing of treatments to pest diel rhythms in activity and susceptibility, however, the current limiting factor is the provision of species-specific interactions and rhythmic data.
2. The literature shows that *P. xylostella*, though already one of the world's most costly lepidopteran pests, is likely to increase in prevalence and damage caused due to climate change, pest treatment resistance and host range expansions, however, also shows potentially exploitable circadian rhythms for the precise timing of IPM.
3. Adult *P. xylostella* express diel rhythmicity in locomotor activity that is expressed nocturnally and highly sensitive to light, appearing to show homeostatic control of total activity while showing no circadian modulation.
4. Adult *P. xylostella* showed rhythmic relative expression of core circadian clock genes despite recorded locomotor data lacking evidence of circadian control, potentially due to the regulation of the specific activity captured.
5. Larval *P. xylostella* showed diel rhythmicity in feeding activity that matched possible host plant signalling rhythms and was maintained into L/L, demonstrating active circadian control of behaviour that gradually damped out in such conditions.
6. Larval *P. xylostella* showed conserved rhythmic expression of core circadian clock genes and showed expression of GSS genes only partially maintained after phase shifts in host plants and therefore is likely also influenced by host plant and environmental conditions. This demonstrates mechanisms for the endogenous maintenance of larval *P. xylostella* feeding rhythms found across conditions and how such may be entrained and limited.

7. OP entrainment and loss of host plant rhythms and defence pathway genes affected the feeding rhythms of *P. xylostella* larvae and choice of host plant leaf tissue, showing the phase of the plant clock and its rhythmicity affected *P. xylostella* feeding. Therefore, though found feeding rhythms are likely under *P. xylostella* circadian clock control, regulation downstream of such interacts with both host plant circadian clocks and expression of defence pathways, potentially relying on JA associated signals.
8. Both adult and larval *P. xylostella* show diel behavioural rhythms, with adults preferentially responding directly to environmental factors, such as light and temperature, while larvae maintain rhythmic entrainment to such into constant conditions. The discussed potential interactions of abiotic factors and host plant circadian clocks and volatiles with *P. xylostella* therefore may be applicable to the enhancement of pest management strategies.

5.3 Future directions

To further this research, both new experiments and extensions to our findings could be carried out with possible options for such detailed below.

- The expression of further clock associated genes could be investigated, such as *cry, Clk* and *Bmal1 (Brain and muscle ARNT-like 1)*/*cyc*. These genes are key components of molecular circadian clocks and will be useful to compare against the expression of *per* and *tim*. *D. plexippus* studies show the presence and activity of these genes, however, it is unclear how they may be expressed in *P. xylostella*, a nocturnal lepidopteran. Other species-specific differences may lead to changes in the timing of expression of clock genes, therefore, it may be informative to investigate any differences between *D. plexippus* and *P. xylostella* in molecular clock gene expression, potentially to infer how evolutionary shifts between diurnal

and nocturnal activity may alter such. These genes, as previously discussed (in: 1.2.2, 5.1.2, 5.1.4), also can have strong associations with light sensitivity and migration (*cry*), and have effects on maintenance of rhythmic defence mechanisms and fitness (Emery et al., 1998, Wijnen and Young, 2006, Zhang et al., 2017, Beaver et al., 2010, Wan et al., 2021). In addition to repeating the experimental procedures carried out for this thesis, on both these extra genes and *per* and *tim*, protein analysis may be useful as there can be differences between translation and transcription levels/protein abundance, specifically under constant conditions, as shown in some previous papers (Greenbaum et al., 2003, Merlin, Gegear and Reppert, 2009). Proteins are the active product of a gene, therefore, understanding how the abundance of this changes over time is highly important for inferring circadian clock function and regulation of resistances for example.

- In addition, tissue-specific qPCR could be carried out on both these clock genes and GSSs. Peripheral clocks may have differing expression levels to CCN and each other, with potentially specific roles in maintaining detoxification, as shown in the midgut, and entrainment responses to feeding (Sauman and Reppert, 1998, Giebultowicz, 2001, Stokkan et al., 2001, Nobata et al., 2012, Bajgar, Jindra and Dolezel, 2013, Niepoth et al., 2018). Therefore, it is also likely to be important to understand the responses in expression of resistance and detoxification genes in these organs, which may be masked using whole-body genetic material. Dissection of *P. xylostella* larvae to remove gut tissues is possible and may be able to address this effectively (Xia et al., 2013, Ma et al., 2018, Yang et al., 2020). Specific CCN can also be isolated as done with *D. plexippus* (Zhu et al., 2008), to carry out similar experiments as described above for qPCR analysis on cells that may inform the circadian regulation of whole *P. xylostella*, identifying a possible source for certain circadian rhythms, such as in reproduction and seeking behaviours.

- However, given appropriate funding, single cell sequencing can be carried out or transcriptomics over time or conditions (Avalos et al., 2019, Tang et al., 2019, Everetts et al., 2021), like those carried out in this thesis. Transcriptomics may also be applied to plant tissues tested to identify whether genes are differentially expressed over time or induced by herbivorous attack (Wang et al., 2020). These experiments and techniques can allow for the matching of both genetic and phenotypic responses between *P. xylostella*, host plants and their circadian rhythms, identifying pathways regulating their interactions. This would allow for further experiments researching the genes identified to be involved in regulating such, potentially identifying key pathways that can be used to enhance herbivory defence or disrupt pest species physiology. Further to this, transcriptomics of the midgut (Perera et al., 2015) can be used to show expression over time in response to host plant material. This may identify regulatory mechanisms for detoxification genes which may be exploitable by timing treatments around changes in expression or inhibiting the regulatory responses themselves. Technologies that can manipulate regulatory responses themselves are becoming more widely available.
- Gene drivers, similar to the GAL4-UAS (Galactose-responsive transcription factor-upstream activating sequence) system, e.g. potentially harnessing a construct using an actin promotor with *Sulf2* (Zhang et al., 2018), may be harnessed to express *P. xylostella* GSS genes, by-passing normal regulatory mechanisms. These methods have been shown to be effective for such across multiple species (Brand and Dormand, 1995, Halpern et al., 2008, Barwell et al., 2017). This can be used to show whether expression of sulfatase genes in *P. xylostella* is a limiting factor of *P. xylostella* herbivory on differing host plants, including mutants affecting glucosinolate production. If increased GSS expression doesn't alter feeding rate and rhythms or growth, then other factors of *P. xylostella* physiology are limiting *P.*

xylostella on specific host plants, therefore, other physiological mechanisms may be more useful to investigate to control *P. xylostella* herbivory. CRISPR-Cas9 (Clustered regularly interspaced short palindromic repeats and CRISPR-associated protein 9) may also be used to edit relative expression levels and rhythms, having been used on many insect species and *P. xylostella* recently (Aumann, Hacker and Schetelig, 2020, Xu et al., 2022), with sex-specific lethality engineered in *P. xylostella* by Oxitec (Jin et al., 2013, Bolton et al. 2019). Knock-out mutants could also be made by such or generated through treatment with GSS siRNA/RNAi (He, Sun and Li, 2012). However, these methods may have issues with additional mutations, non-target effects and incomplete repression, making it more difficult to make clear conclusions. Modified CRISPR-Cas9 (Cas9/sgRNA) may be used on *P. xylostella* to increase the effectiveness of these experimental pathways (Ren et al., 2013). These methods could further show how regulation of GSS genes modulates feeding activity rhythms and may demonstrate whether GSS expression is a key limiting factor of both *P. xylostella* brassica feeding and entrainment to host-plant circadian rhythms and natural plant defences.

- In addition to *P. xylostella* mutants, further *A. thaliana* mutant experiments may be useful. The removal of aliphatic (*myb28/29*) and indolic (*cyp79B2/B3*) glucosinolates has been achieved in *A. thaliana* mutants (Mostafa et al., 2018) and can be used to identify the specific glucosinolate *P. xylostella* may respond to or entrain their activity and molecular expression to, including how their presence limits attraction and feeding. This will identify the specific natural plant defence pathway that can be best exploited to reduce *P. xylostella* feeding, whether through herbivory defence or reduced attraction.
- qPCR studies on used *B. rapa* and *A. thaliana* in our experiments would be useful for comparing the molecular expression of plant clock and defence pathways genes

to the *P. xylostella* molecular and behavioural responses. Allowing the responses to be matched up to identify molecular interactions between *P. xylostella* and host plants. Direct measurements of glucosinolate concentrations in such plants (Goodspeed et al., 2013b, Doghri et al., 2022) would also allow for direct comparisons between glucosinolate presence, feeding and GSS responses in *P. xylostella*. This can also include the previously mentioned *myb28/29* and *cyp79B2/B3* mutants, also categorising plant volatile compound excretions (Barbosa-Cornelio et al., 2019), potentially isolating those that most effect *P. xylostella* and herbivory defence to enhance natural plant defences.

- Extensions to our adult behavioural assays could also be made, such as exposing group populations of *P. xylostella* to further L/D conditions like done for locomotor assays and with semi-field conditions. Any further differences found we can infer are due to the features of these setup where *P. xylostella* could fly freely and interact with each other or have ramping lights and temperature cycles. Plants could be provided in the population cage setup also, though taking up more space, any changes in adult *P. xylostella* activity can be attributed to volatile signals coming the plant host, identifying how plant hosts can influence *P. xylostella* behaviour. Further semi-field conditions could also be used, maintaining more extreme light and temperature profiles such as UK August and October used by Shaw et al. 2019, or incorporating other abiotic or biotic factors, such as humidity spikes or predation signals (Kronfeld-Schor et al., 2017, Shaw et al. 2019). This will allow us to more accurately apply recorded *P. xylostella* activity across more environmental conditions. It may also be useful to carry out our larval experiments under similar conditions to the adult *P. xylostella*. Including exposing feeding assays to semi-field conditions or varying light cycles, or loading larvae into locomotor activity monitors. This would provide more information on the activity of *P. xylostella*

larvae directly and support the data from other experimental setups. In addition, colonies could be entrained over periods of time to the experimental conditions then released into constant condition. This will show how the combination of environmental factors can lead to variations in *P. xylostella* activity and amount of herbivory on specific plant materials, potentially better identifying the responses of *P. xylostella* in the wild and on crops.

- Isolating temperature effects may also be a simple addition, maintaining L/L and D/D conditions with specific temperature profiles then also removing such to monitor how activity may be entrained and regulated by temperature, helping further predict *P. xylostella* responses in the wild and potentially to climate change.
- Rearing our used strain on arrhythmic substrate through successful artificial diet may also address any possible effect of prior host plant interactions and entrainment effects on adult behavioural data (Pivnick et al., 1990, Miranda-Anaya, Guevara-Fefer and Garcia-Rivera, 2002, Grayson et al., 2015), recording *P. xylostella* activity when exposed to novel host plants or *B. rapa*. Similar to Wybouw et al., 2015's findings that interactions with prior hosts caused significantly increased changes in expression (Wybouw et al., 2015).
- Further to this, Geneva 88 strain and WT collections of *P. xylostella* could also be compared, potentially highlighting differences between long-term lab reared strains on either *B. rapa* or artificial diet and current WT populations infesting brassica crops. If possible this may also be extended to include pea-adapted strains (Knolhoff and Heckel, 2011, Henniges-Janssen et al., 2011a,b). This will hopefully help better show how wild *P. xylostella* populations may behave and interact with host plants and their defences, potentially being extended using above-mentioned methods to uncover any shifts in molecular expression. Additionally shedding light

on how specific lab environment rearing can cause changes in species, specifically their circadian entrainment and responses.

- In addition, maintenance of *P. xylostella* larvae on *A. thaliana* or artificial diet for multiple generations or including new strains as mentioned above, could also allow for better comparisons. This would show possible interaction effects of prior entrainment and direct responses to environmental factors such as plant volatiles, where full prior entrainment may take more time or multiple generations for a species to adapt to fit new host plant. (Lohr and Gathu, 2011, Grayson et al., 2015, Wybouw et al., 2015, Hoffmann and Ross, 2018).
- Extensions to larval *P. xylostella* assays may be useful as re-entrainment to shifted host plant circadian clocks such as in OP conditions can take multiple days (Goodspeed et al., 2013b), therefore extending continuous data collections beyond 24h or increasing length in L/L conditions may be useful. This would further show if *P. xylostella* entrain to OP host plants over time, demonstrating a mechanism by which *P. xylostella* modulates their behaviour to match possible host plants.
- Keeping host plants IP with abiotic zeitgeber signals and having *P. xylostella* as the 'time travellers' may also provide important information on the interaction between abiotic and host plant signals on defining *P. xylostella* larval feeding rhythms. Similar can be said with both these points for the mutant *A. thaliana* assays, including carrying out choice assays for plant defence mutants and qPCR analysis on mutant *A. thaliana* fed *P. xylostella*.
- Keeping host plants IP with abiotic zeitgeber signals and having *P. xylostella* as the 'time travellers', by moving 12h phase advanced *P. xylostella* larvae to repeat light phase or dark phase on OP host plant material, may also provide important information on the interaction between abiotic and host plant signals on defining *P. xylostella* larval feeding rhythms as both are now OP with *P. xylostella*, allowing

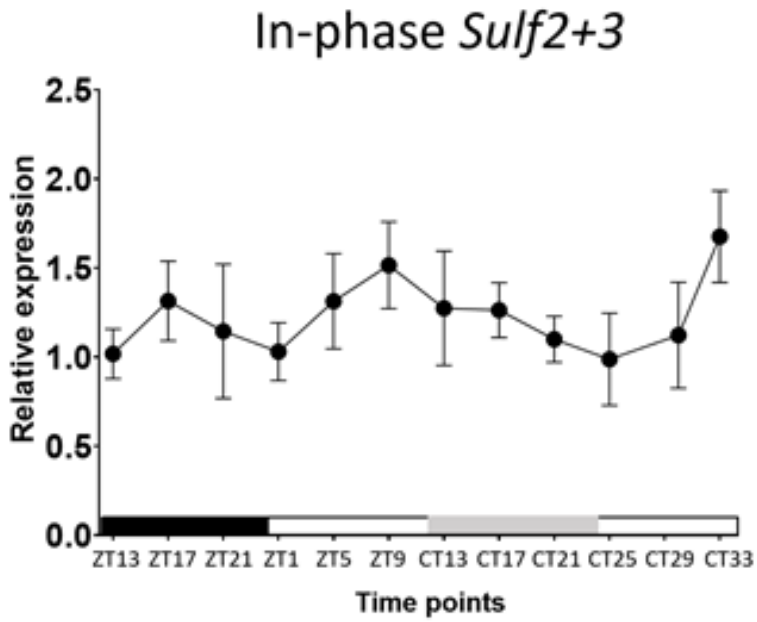
for comparison with *P. xylostella* results where only the plant host signals where OP. Potentially showing which signals *P. xylostella* is mostly reacting to. If there is no difference in the results of the experiments, then *P. xylostella* is mostly reacting to the OP host plant volatiles rather than light cycle. Similar can be said for the mutant *A. thaliana* assays.

- Further choice assays for plant defence mutants can be used to show difference in attraction levels of mutant leaves with mutations at specific points along JA pathways. This will help identify which parts of this pathway is responsible for producing volatiles *P. xylostella* is attracted too, such as glucosinolates. Carrying out qPCR analysis on mutant *A. thaliana* fed *P. xylostella* will also show how GSSs are expressed in response to these mutans plants compared to WT *A. thaliana* and the WT *B. rapa* results.
- Appropriate calculations of endogenous period length in insects after species are released into constant conditions requires at least 5 days of data collection for reliable calculations (Tataroglu and Emery, 2014). Therefore 6 days of continuous exposure to L/L for *P. xylostella* larval feeding may be attempted, similar to adult locomotor assays and potentially using a rotation of new leaves and using younger/earlier instar *P. xylostella* larvae, to calculate and monitor changes to *P. xylostella* larval endogenous circadian clock regulation through period length changes. This will confirm the loss of or any shifts in *P. xylostella* larvae by allowing the use of period statistics such as periodogram analysis. However, damping of rhythmicity may prevent period length calculations, yet if carried out at the molecular level over time (using qPCR), past L/L1, the molecular run-down of the circadian clock can be proven to be occurring in *P. xylostella*, rather than in the plant (Millar et al., 1995, Thain et al., 2002) by following the expression of clock genes such as *per* and *tim*. Arrhythmicity by day 3 in L/L (L/L3) can be further

tested by extending into deeper L/L conditions to check if this is permeant or if re-entrainment occurs. Artificial diet may allow for experiments in D/D for larval feeding, which should allow for good comparisons between the effect of L/L on feeding as well as comparing against differences between feeding rhythms on arrhythmic substate and host plants highlighting the effects of associated volatile signals.

- Chronotoxicity experiments may be carried out directly to test whether important detoxification rhythms, co-opted for pesticide resistance (Tao et al., 2012), cause rhythms in pesticide toxicity, potentially being least resistant before light phase onset when GSS expression may be low as we have shown. Peak *P. xylostella* feeding, occurring around dark phase onset, may also lead to increased uptake of control treatments, potentially increasing the efficacy of such around this time. Further IPM strategies shown previously (in: 1.3.5, 5.1.6) may also be trialled on both *P. xylostella* adults and larvae, such as biological control agents and through reproductive and development disruptions. Time-dependent susceptibility of gut microbiota to treatments, important due to provision of resistances and digestion, may also be present (Xia et al., 2013, Xia et al., 2018, Yang et al., 2020). By investigating how *P. xylostella* interact with these treatments, and if there is rhythmic susceptibility in such, the efficacy of *P. xylostella* management strategies may be increased while reducing cost and damages to the environment.

Appendix A *Sulf2+3* supplemental



Supplementary Figure 1 In-phase qPCR relative expression of *Sulf2+3* amplicon.

Larval *P. xylostella* rhythmic mRNA expression levels of an amplicon matching both *Sulf2* and *Sulf3* maintains similar rhythmicity to individual GSS genes in both L/D and L/L feeding conditions. Variations between both genex relative expression levels however likely lead to reduced rhythmic amplitude and increased error. Line graph with SEM error bars showing such for GSS genes, with 12/12 L/D zeitgeber time denoted by ZT time points and a light grey bar for circadian dark phase and CT time points in L/L.

Transcript	Rhythm *P*	Mesor	Amplitude	Phase peak	ANOVA 24h
Sulf2+3	4.5^{-2}	1.23	0.17	10.41	*P >0.05*

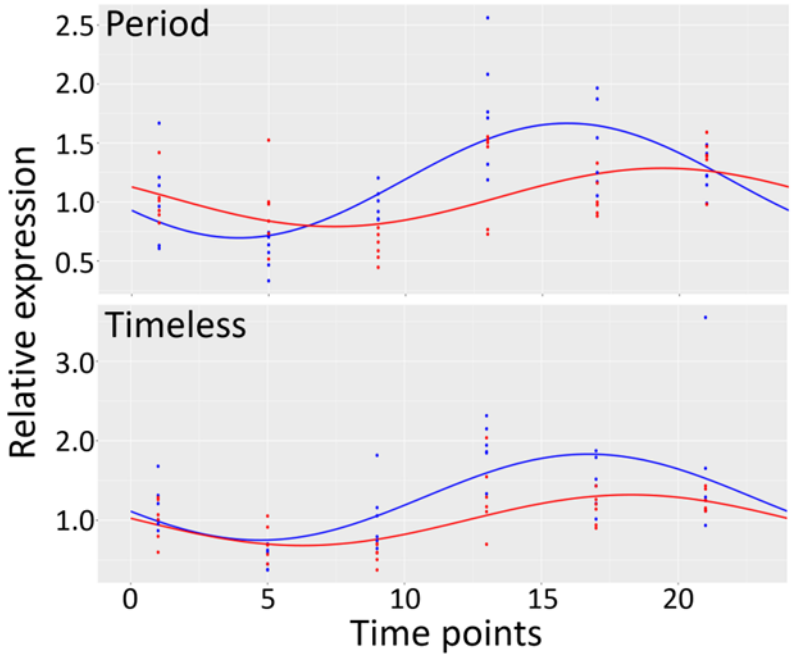
ANOVA	*Sulf2+3* L/D	*Sulf2+3* L/L
P value	*P* >0.05	*P* >0.05
Time points	Sig	Sig
ZT1/CT1		
ZT5/CT5		
ZT9/CT9		
ZT13/CT13		
ZT17/CT17		
ZT21/CT21		

2-way ANOVA	*Sulf2+3* L/D vs D/D %	*P* value
Condition	n/a	*P >0.05*
Time	n/a	*P >0.05*
Interaction	n/a	*P >0.05*

Supplementary Table 1 Statistical tests of Sulf2+3 amplicon relative expression.

CircaCompare results from 48h rhythms of *Sulf2+3* relative expression levels are shown along with Kruskal-Wallis tests of 24h collated rhythms and comparisons between L/D and L/L day time points. 2-way ANOVA results also shown for combination between effects of time over 24h day, whether such is in L/D or L/L conditions and whether there is any significant interaction between the two variables. 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 (s) dark phase onset is the peak of fitted c

Appendix B CircaCompare supplementals



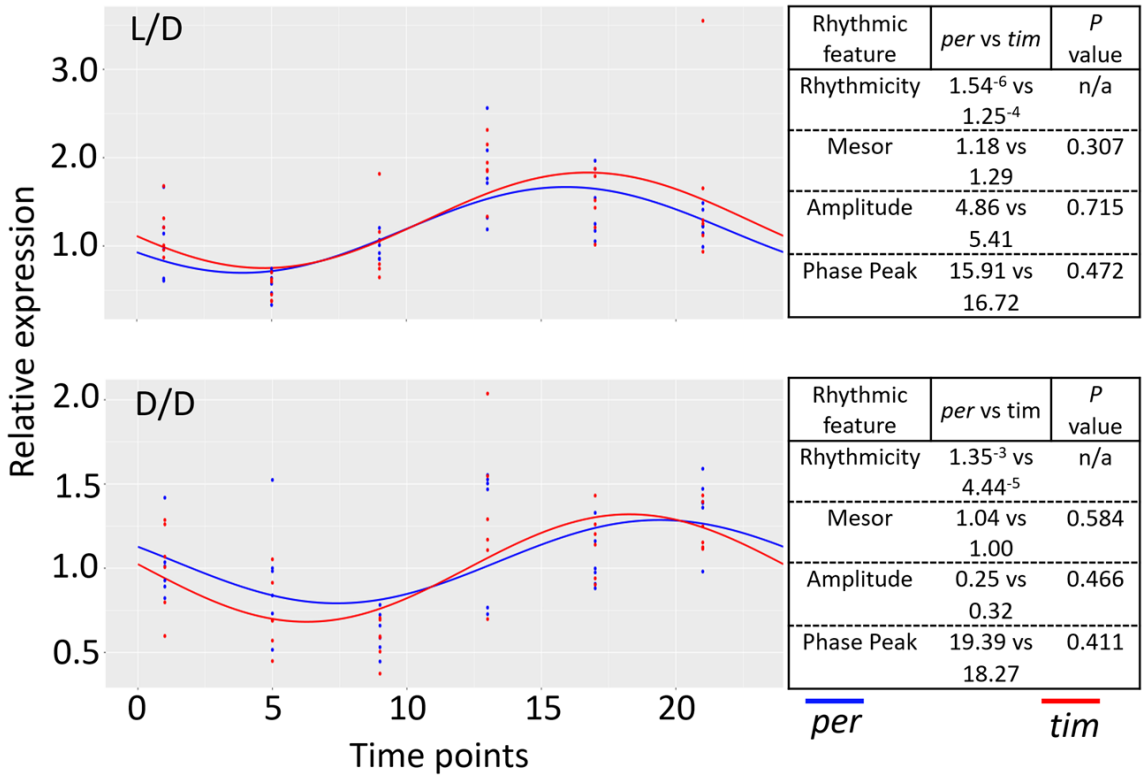
Rhythmic feature	L/D vs D/D	*P* value
Rhythmicity	1.54^{-6} vs 1.35^{-3}	n/a
Mesor	1.18 vs 1.04	0.070
Amplitude*	0.49 vs 0.25	0.032
Phase Peak*	15.90 vs 19.40	0.011

Rhythmic feature	L/D vs D/D	*P* value
Rhythmicity	1.25^{-4} vs 4.44^{-5}	n/a
Mesor*	1.29 vs 1.00	0.005
Amplitude	0.54 vs 0.32	0.122
Phase Peak	16.72 vs 18.27	0.269

L/D D/D

Supplementary Figure 2 CircaCompare analysis of adult male *P. xylostella per* and *tim* expression.

CircaCompare cosinusoidal curve fitting against individual data points of *per* and *tim* data from Fig. 3.14 compared between L/D and D/D conditions. *P* values for comparisons between rhythmic features of curve shown in respective tables to right of plots. Relative expression levels calculated from qPCR data using the 2–ΔΔCt method and * against feature name indicates significant differences between L/D and D/D conditions. Rhythmicity *P* value shown in middle column, 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. Time points refer to L/D zeitgeber time (ZT) and D/D circadian time (CT) time points with phase peak equating to such respectively.

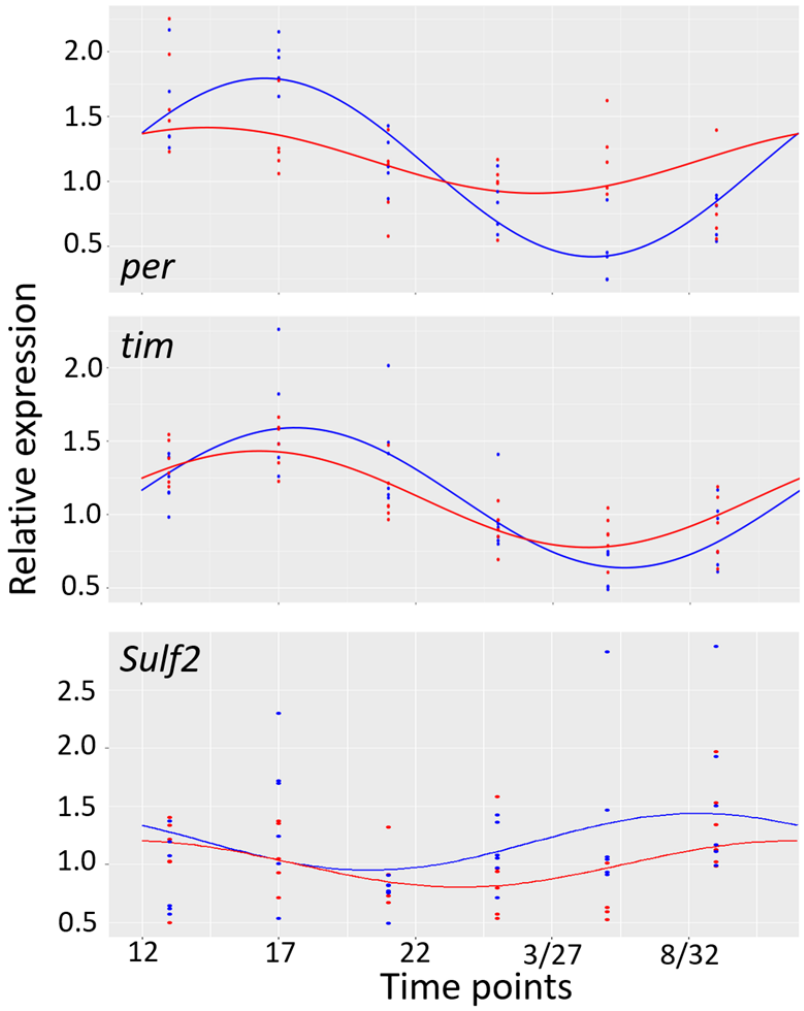


Rhythmic feature	*per* vs *tim*	*P* value
Rhythmicity	1.54^{-6} vs 1.25^{-4}	n/a
Mesor	1.18 vs 1.29	0.307
Amplitude	4.86 vs 5.41	0.715
Phase Peak	15.91 vs 16.72	0.472

Rhythmic feature	*per* vs tim	*P* value
Rhythmicity	1.35^{-3} vs 4.44^{-5}	n/a
Mesor	1.04 vs 1.00	0.584
Amplitude	0.25 vs 0.32	0.466
Phase Peak	19.39 vs 18.27	0.411

Supplementary Figure 3 CircaCompare analysis between *per* and *tim* relative expression in adult male *P. xylostella* in L/D and D/D conditions.

CircaCompare cosinusoidal curve fitting against individual data points of adult male *P. xylostella per* and *tim* data from Fig. 3.14 compared together in L/D and D/D conditions. *P* values for comparisons between rhythmic features of curve shown in respective tables to right of plots. Relative expression levels calculated from qPCR data using the 2–ΔΔCt method. Time points refer to ZT and CT time points with phase peak equating to such respectively.



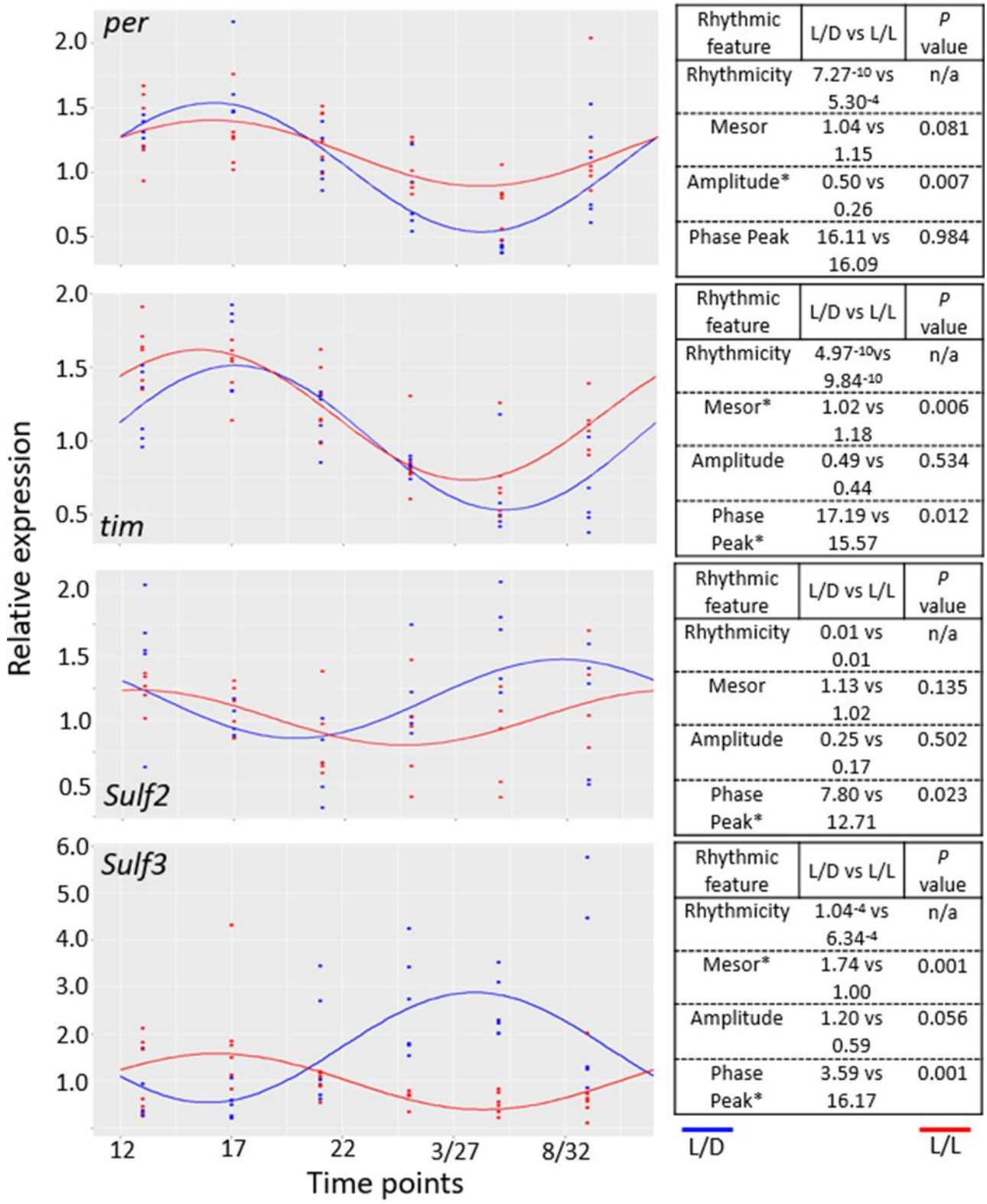
Rhythmic feature	L/D vs L/L	*P* value
Rhythmicity	1.37^{-10} vs 0.015	n/a
Mesor	1.11 vs 1.16	0.519
Amplitude*	0.69 vs 0.25	0.001
Phase Peak	16.48 vs 14.37	0.124

Rhythmic feature	L/D vs L/L	*P* value
Rhythmicity	2.03^{-9} vs 5.03^{-9}	n/a
Mesor	1.11 vs 1.10	0.846
Amplitude*	0.48 vs 0.33	0.043
Phase Peak	17.60 vs 16.29	0.072

Rhythmic feature	L/D vs L/L	*P* value
Rhythmicity	0.070 vs 0.014	n/a
Mesor	1.19 vs 1.00	0.079
Amplitude	0.24 vs 0.20	0.762
Phase Peak	8.34 vs 35.67	0.217

Supplementary Figure 4 CircaCompare analysis of in-phase larval *P. xylostella per, tim* and *Sulf2* relative expression.

CircaCompare cosinusoidal curve fitting against individual data points of in-phase *per, tim* and *Sulf2* data from Fig. 4.3 compared between L/D and L/L conditions. *P* values for comparisons between rhythmic features of curve shown in respective tables to right of plots. Relative expression levels calculated from qPCR data using the 2–ΔΔCt method and * against feature name indicates significant differences between L/D and L/L conditions. Of GSS genes only *Sulf2* L/L condition relative expression was calculated as rhythmic by CircaCompare analysis. Rhythmicity *P* value shown in middle column, 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. Time points refer to L/D zeitgeber time (ZT) and L/L circadian time (CT) time points with phase peak equating to such respectively.



Rhythmic feature	L/D vs L/L	*P* value
Rhythmicity	7.27^{-10} vs 5.30^{-4}	n/a
Mesor	1.04 vs 1.15	0.081
Amplitude*	0.50 vs 0.26	0.007
Phase Peak	16.11 vs 16.09	0.984

Rhythmic feature	L/D vs L/L	*P* value
Rhythmicity	4.97^{-10}vs 9.84^{-10}	n/a
Mesor*	1.02 vs 1.18	0.006
Amplitude	0.49 vs 0.44	0.534
Phase Peak*	17.19 vs 15.57	0.012

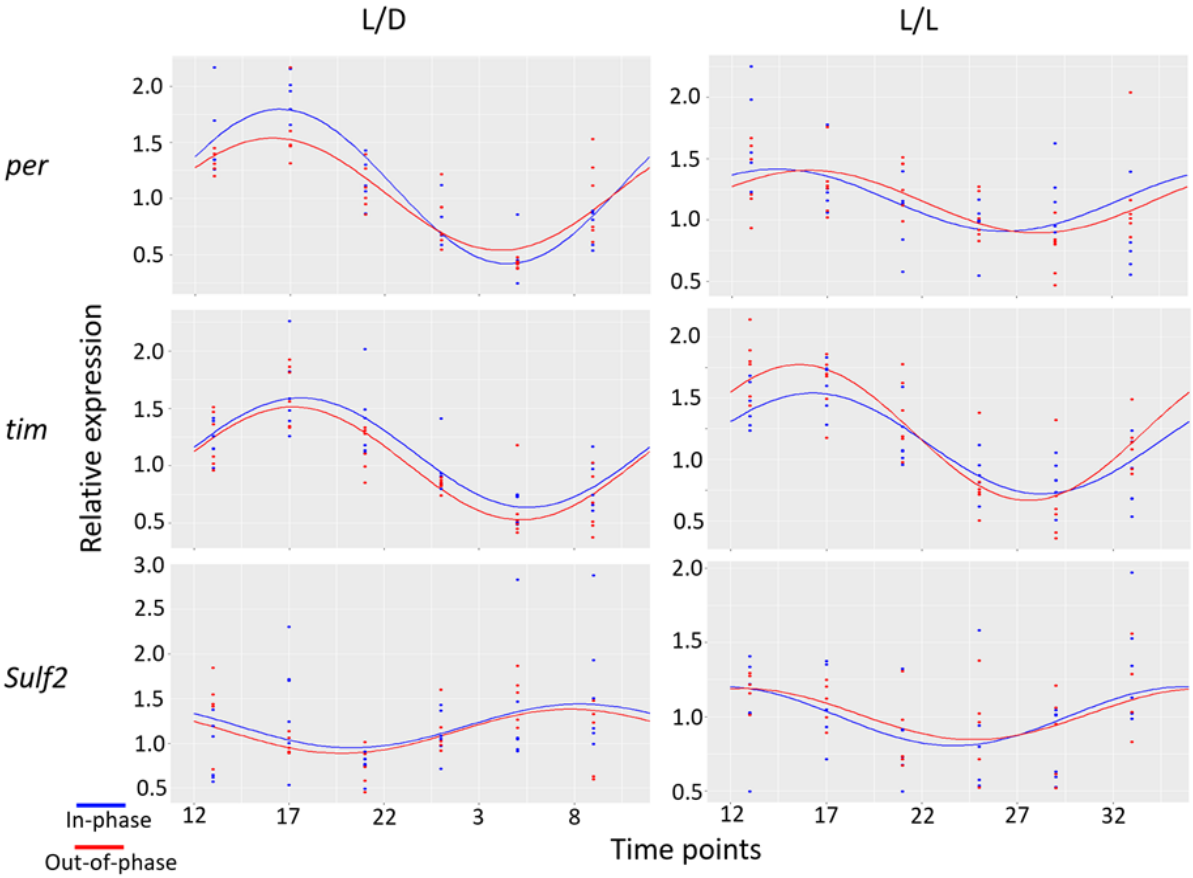
Rhythmic feature	L/D vs L/L	*P* value
Rhythmicity	0.01 vs 0.01	n/a
Mesor	1.13 vs 1.02	0.135
Amplitude	0.25 vs 0.17	0.502
Phase Peak*	7.80 vs 12.71	0.023

Rhythmic feature	L/D vs L/L	*P* value
Rhythmicity	1.04^{-4} vs 6.34^{-4}	n/a
Mesor*	1.74 vs 1.00	0.001
Amplitude	1.20 vs 0.59	0.056
Phase Peak*	3.59 vs 16.17	0.001

Supplementary Figure 5 CircaCompare analysis of out-of-phase larval *P. xylostella* relative expression.

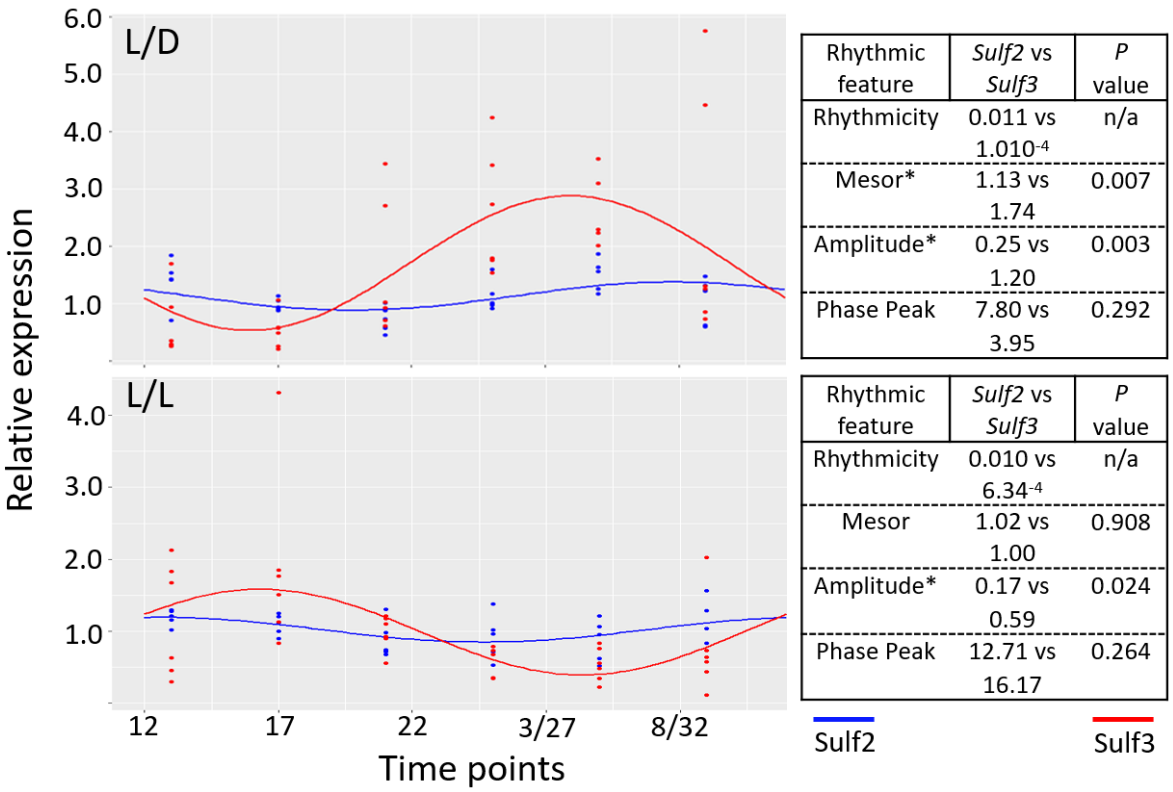
CircaCompare cosinusoidal curve fitting against individual data points of out-of-phase *per, tim*, *Sulf2* and *Sulf3* data from Fig. 4.3 compared between L/D and L/L conditions. *P* values for comparisons between rhythmic features of curve shown in

respective tables to right of plots. Relative expression levels calculated from qPCR data using the 2–ΔΔCt method and * against feature name indicates significant differences between L/D and L/L conditions. Time points refer to ZT/CT with phase peak equating to such respectively.



Supplementary Figure 6 CircaCompare analysis between in and out-of-phase relative expression in L/D and L/L conditions.

CircaCompare cosinusoidal curve comparison of data points from in and out-of-phase *per, tim* and *Sulf2* results shown in Fig. 4.3 compared between eachother in L/D and L/L conditions. Time points referring to ZT in L/D and CT in L/L. Data in Table 4.5.

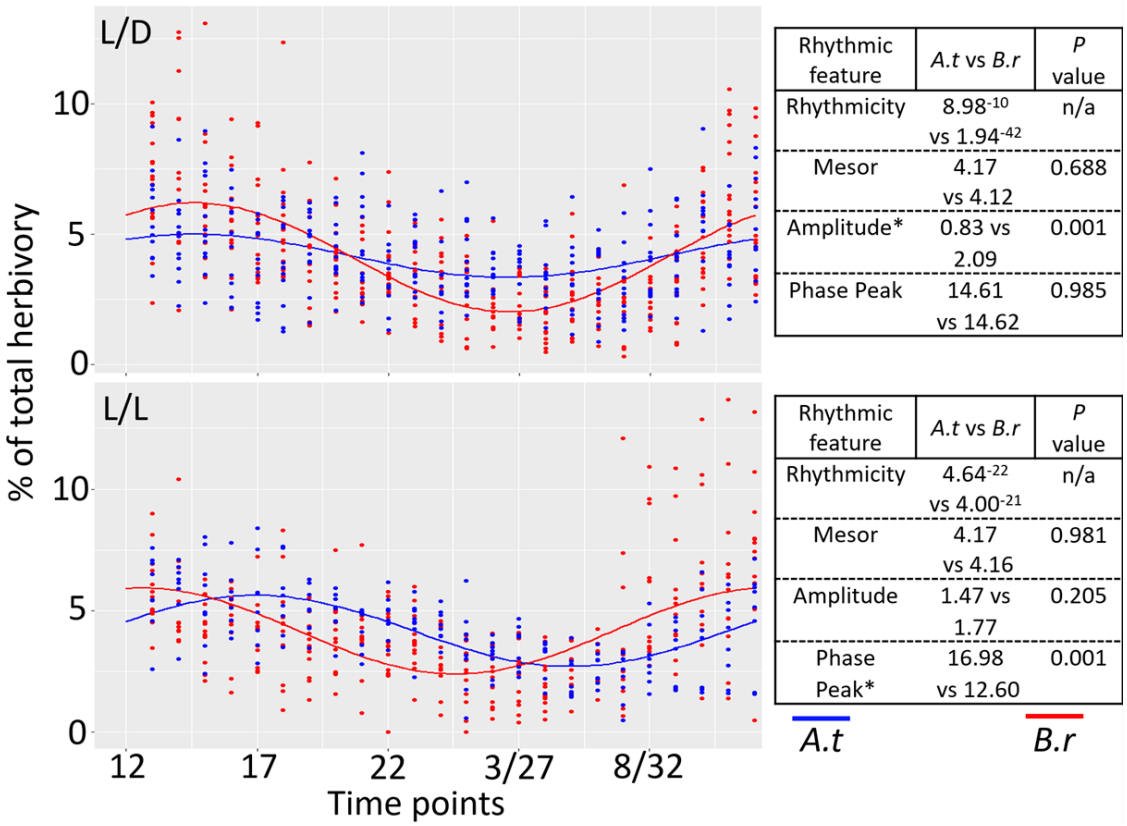


Rhythmic feature	*Sulf2* vs *Sulf3*	*P* value
Rhythmicity	0.011 vs 1.010^{-4}	n/a
Mesor*	1.13 vs 1.74	0.007
Amplitude*	0.25 vs 1.20	0.003
Phase Peak	7.80 vs 3.95	0.292

Rhythmic feature	*Sulf2* vs *Sulf3*	*P* value
Rhythmicity	0.010 vs 6.34^{-4}	n/a
Mesor	1.02 vs 1.00	0.908
Amplitude*	0.17 vs 0.59	0.024
Phase Peak	12.71 vs 16.17	0.264

Supplementary Figure 7 CircaCompare analysis comparison of out-of-phase GSS gene relative expression.

CircaCompare cosinusoidal curve fitting against individual data points of out-of-phase *Sulf2* and *Sulf3* data from Fig. 4.3 compared between eachother in L/D and L/L conditions. *P* values for comparisons between rhythmic features of curve shown in respective tables to right of plots. Relative expression levels calculated from qPCR data using the 2–ΔΔCt method and * against feature name indicates significant differences between *Sulf2* and *Sulf3* relative expression rhythms. Time points refer to ZT/CT with phase peak equating to such respectively.



Rhythmic feature	*A.t* vs *B.r*	*P* value
Rhythmicity	8.98^{-10} vs 1.94^{-42}	n/a
Mesor	4.17 vs 4.12	0.688
Amplitude*	0.83 vs 2.09	0.001
Phase Peak	14.61 vs 14.62	0.985

Rhythmic feature	*A.t* vs *B.r*	*P* value
Rhythmicity	4.64^{-22} vs 4.00^{-21}	n/a
Mesor	4.17 vs 4.16	0.981
Amplitude	1.47 vs 1.77	0.205
Phase Peak*	16.98 vs 12.60	0.001

Supplementary Figure 8 CircaCompare analysis of *A.t* vs *B.r* feeding rhythms.

CircaCompare cosinusoidal curve comparison of % of total herbivory over time between WT *A. thaliana* (*A.t*) and *B. rapa* (*B.r*) *P. xylostella* larval feeding. Data points from Fig. 4.1 and Fig. 4.5. Time points refer to ZT/CT with phase peak equating to such. * against feature name indicates significant differences between *A. thaliana* and *B. rapa* feeding.

WT L/D VS L/L

Rhythmic feature	L/D vs L/L	*P* value
Rhythmicity	8.97^{-10} vs 4.64^{-22}	n/a
Amplitude*	0.83 vs 1.47	0.001
Phase Peak*	14.61 vs 16.98	0.001

WT L/D VS mutant L/D

Rhythmic feature	WT vs CCA1-ox	*P* value	Rhythmic feature	WT vs TM	*P* value
Rhythmicity	8.97^{-10} vs 8.53^{-05}	n/a	Rhythmicity	8.97^{-10} vs 3.04^{-4}	n/a
Amplitude	0.83 vs 0.91	0.730	Amplitude	0.83 vs 0.56	0.191
Phase Peak*	14.61 vs 18.94	0.001	Phase Peak*	14.61 vs 18.73	0.001

Rhythmic feature	WT vs *aos*	*P* value	Rhythmic feature	WT vs *opr3*	*P* value	Rhythmic feature	WT vs *coi1-16*	*P* value
Rhythmicity	8.97^{-10} vs 2.29^{-14}	n/a	Rhythmicity	8.97^{-10} vs 4.06^{-3}	n/a	Rhythmicity	8.97^{-10} vs 1.40^{-10}	n/a
Amplitude*	0.83 vs 1.43	0.005	Amplitude	0.83 vs 0.53	0.150	Amplitude*	0.83 vs 1.32	0.031
Phase Peak*	14.61 vs 17.99	0.001	Phase Peak	14.61 vs 16.87	0.100	Phase Peak*	14.61 vs 18.27	0.001

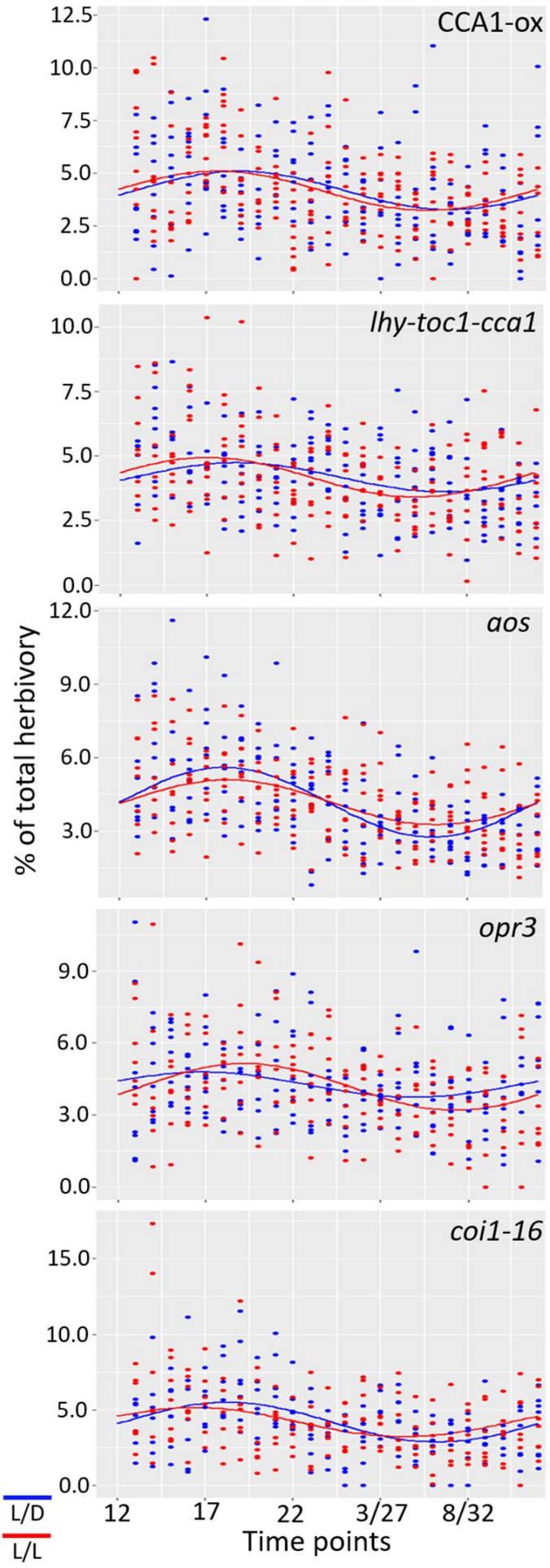
WT L/L VS mutant L/L

Rhythmic feature	WT vs CCA1-ox	*P* value	Rhythmic feature	WT vs TM	*P* value
Rhythmicity	4.64^{-22} vs 4.13^{-6}	n/a	Rhythmicity	4.64^{-22} vs 1.35^{-5}	n/a
Amplitude*	1.47 vs 0.93	0.026	Amplitude*	1.47 vs 0.76	0.001
Phase Peak	16.98 vs 17.68	0.395	Phase Peak	16.98 vs 17.04	0.938

Rhythmic feature	WT vs *aos*	*P* value	Rhythmic feature	WT vs *opr3*	*P* value	Rhythmic feature	WT vs *coi1-16*	*P* value
Rhythmicity	4.64^{-22} vs 3.48^{-8}	n/a	Rhythmicity	4.64^{-22} vs 1.82^{-6}	n/a	Rhythmicity	4.64^{-22} vs 2.27^{-4}	n/a
Amplitude*	1.47 vs 0.92	0.008	Amplitude*	1.47 vs 0.98	0.035	Amplitude	1.47 vs 0.96	0.072
Phase Peak	16.98 vs 18.21	0.080	Phase Peak*	16.98 vs 19.27	0.003	Phase Peak	16.98 vs 16.39	0.533

Supplementary Table 2 Col-0 *A. thaliana* CircaCompare comparison with mutant *A. thaliana*.

CircaCompare cosinusoidal curve fitting results from comparisons between Col-0 WT *A. thaliana* and mutant *A. thaliana* in both L/D and L/L conditions individually. Phase peak equates to ZT/CT time points. TM refers to triple mutant (*cca1-11/lhy-21/toc1-21)*, * against feature name indicates significant differences between WT and mutant in respective conditions.


CCA1-ox
lhy-toc1-cca1
aos
opr3
coi1-16
% of total herbivory
Time points
12
17
22
3/27
8/32
L/D
L/L

Supplementary Figure 9 CircaCompare analysis between mutant *A. thaliana* L/D and L/L conditions.

CircaCompare cosinusoidal curve comparison of % of total herbivory over time between mutant *A. thaliana* in L/D and L/L condition *P. xylostella* larval feeding. Time points referring to ZT/CT. Data in table 4.7, no significant differences in mesor.

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