IS THE PHENOLOGY OF PEA APHIDS (*ACYRTHOSIPHON PISUM*) CONSTRAINED BY DIURNAL RHYTHMS?

WIRD DIE PHÄNOLOGIE DER ERBSENBLATTLAUS (*ACYRTHOSIPHON PISUM*) DURCH TAG-/NACHTRHYTHMIK LIMITIERT?

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Whoever looks at the insect world, at flies, aphides, gnats, and innumerable parasites, and even at the infant mammals, must have remarked the extreme content they take in suction, which constitutes the main business of their life. If we go into a library or news-room, we see the same function on a higher plane, performed with like ardor, with equal impatience of interruption, indicating the sweetness of the act.

Ralph Waldo Emerson, The Complete Works

Aphis sedi nymph, sucking from its host plant shortly after emerging.

AFFIDAVIT

I hereby confirm that my thesis entitled "**Is the phenology of pea aphids (*Acyrthosiphon pisum*) constrained by diurnal rhythms?**" is the result of my own work. I did not receive any help or support from commercial consultants. All sources and / or materials applied are listed and specified in the thesis.

Furthermore, I confirm that this thesis has not yet been submitted as part of another examination process neither in identical nor in similar form.

Place, Date Signature

EIDESSTATTLICHE ERKLÄRUNG

Hiermit erkläre ich an Eides statt, die Dissertation „**Wird die Phänologie der Erbsenblattlaus (*Acyrthosiphon pisum*) durch Tag-/Nachtrhythmik limitiert**?" eigenständig, d.h. insbesondere selbständig und ohne Hilfe eines kommerziellen Promotionsberaters, angefertigt und keine anderen als die von mir angegebenen Quellen und Hilfsmittel verwendet zu haben.

Ich erkläre außerdem, dass die Dissertation weder in gleicher noch in ähnlicher Form bereits in einem anderen Prüfungsverfahren vorgelegen hat.

Ort, Datum Unterschrift

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SUMMARY

The rotation of the earth leads to a cyclic change of night and day. Numerous strategies evolved to cope with diurnal change, as it is generally advantageous to be synchronous to the cyclic change in abiotic conditions. Diurnal rhythms are regulated by the circadian clock, a molecular feedback loop of RNA and protein levels with a period of circa 24 hours. Despite its importance for individuals as well as for species interactions, our knowledge of circadian clocks is mostly confined to few model organisms.

While the structuring of activity is generally adaptive, a rigid temporal organization also has its drawbacks. For example, the specialization to a diurnal pattern limits the breadth of the temporal niche. Organisms that are adapted to a diurnal life style are often poor predators or foragers during night time, constraining the time budget to only diurnal parts of the day/night cycle.

Climate change causes shifts in phenology (seasonal timing) and northward range expansions, and changes in season or in latitude are associated with novel day length – temperature correlations. Thus, seasonal organisms will have some life history stages exposed to novel day lengths, and I hypothesized that the diurnal niche determines whether the day length changes are beneficial or harmful for the organism. I thus studied the effects of day length on life-history traits in a multi-trophic system consisting of the pea aphid *Acyrthosiphon pisum* and predatory larvae of *Chrysoperla carnea* (common green lacewing) and *Episyrphus balteatus* (marmalade hoverfly). In order to identify the mechanisms for phenological constraints I then focused on diurnal rhythms and the circadian clock of the pea aphid.

Aphids reacted to shorter days with a reduced fecundity and shorter reproductive period. Short days did however not impact population growth, because the fitness constraints only became apparent late in the individual's life. In contrast, *E. balteatus* grew 13% faster in the shorter day treatment and preyed on significantly more aphids, whereas *C. carnea* grew 13% faster under longer days and the elevation of predation rates was marginally significant. These results show that day length affects vital life-history traits, but that the direction and effect size depends on species.

I hypothesized that the constraints or fitness benefits are caused by a constricted or expanded time budget, and hence depend on the temporal niche. *E. balteatus* is indeed night-active and *C. carnea* appears to be crepuscular, but very little data exists for *A. pisum*. Hence, I reared the pea aphid on an artificial diet and recorded survival, moulting and honeydew excretion. The activity patterns were clearly rhythmic and molting and honeydew excretion were elevated during day-time. Thus, the diurnal niche could explain the observed, but weak, day length constraints of aphids.

The diurnal niche of some organisms is remarkably flexible, and a flexible diurnal niche may explain why the day length constrains were relatively low in *A. pisum*. I thus studied its circadian clock, the mechanism that regulates diurnal rhythms. First, I improved an artificial diet for *A. pisum*, and added the food colorant Brilliant Blue FCF. This food colorant stained gut and

honeydew in low concentration without causing mortalities, and thus made honeydew excretion visible under dim red light. I then used the blue diet to raise individual aphids in 16:08 LD and constant darkness (DD), and recorded honeydew excretion and molting under red light every three hours. In addition, we used a novel monitoring setup to track locomotor activity continuously in LD and DD. Both the locomotor rhythm and honeydew excretion of *A. pisum* appeared to be bimodal, peaking in early morning and in the afternoon in LD. Both metabolic and locomotor rhythm persisted also for some time under constant darkness, indicating that the rhythms are driven by a functional circadian clock. However, the metabolic rhythm damped within three to four days, whereas locomotor rhythmicity persisted with a complex distribution of several free-running periods. These results fit to a damped circadian clock that is driven by multiple oscillator populations, a model that has been proposed to link circadian clocks and photoperiodism, but never empirically tested.

Overall, my studies integrate constraints in phenological adaptation with a mechanistic explanation. I showed that a shorter day length can constrain some species of a trophic network while being beneficial for others, and linked the differences to the diurnal niche of the species. I further demonstrated that a flexible circadian clock may alleviate the constraints, potentially by increasing the plasticity of the diurnal niche.

ZUSAMMENFASSUNG (GERMAN)

Die Rotation der Erde bedingt den zyklischen Wechsel von Tag und Nacht. Verschiedene Anpassungen an den täglichen Wechsel evolvierten, da es generell von Vorteil ist, mit der abiotischen Umwelt synchron zu sein. Die Tagesrhythmik wird von der circadianen Uhr reguliert, einem molekularen Rückkopplungsmechanismus auf RNA- und Protein- Ebene mit einer Periode von etwa 24 Stunden. Trotz der Bedeutung der circadianen Uhr, sowohl für Individuen als auch für Wechselwirkungen mit anderen Arten, ist unser Wissen auf wenige Modellorganismen beschränkt.
Während die Strukturierung von Aktivitätsmustern im Wesentlichen adaptiv ist, kann eine strenge zeitliche Organisation auch Nachteile mit sich bringen. Zum Beispiel limitiert die Spezialisierung auf ein Aktivitätsmuster die Breite der zeitlichen Nische. So können tagaktive Organismen häufig nur schlecht in Dunkelheit Nahrung finden, so dass das Zeitbudget von der Tageszeit begrenzt wird.
Der Klimawandel führt zu Veränderungen der Phänologie (saisonales Timing) und zur Ausbreitung der Arten Richtung Norden, und Veränderungen in der Phänologie oder im Breitengrad sind mit neuen Korrelationen von Tageslänge und Temperatur verknüpft. Daher werden einige Stadien im Lebenszyklus saisonaler Organismen neuen Tageslängen ausgesetzt. Ich habe die Hypothese aufgestellt, dass die zeitliche Nische bestimmt, ob Veränderungen in der Tageslänge für den Organismus von Vorteil oder von Nachteil sind. Daher untersuchte ich die Effekte von Tageslängen auf den Lebenszyklus von Arten in einem multi-trophischen System, bestehend aus der Erbsenblattlaus, *Acyrthosiphon pisum* und räuberisch lebenden Larven von *Chrysoperla carnea* (Gemeine Florfliege) und *Episyrphus balteatus* (Hainschwebfliege). Um die Mechanismen der Einschränkungen in der Phänologie zu verstehen, untersuchte ich anschließend die Tagesrhythmik und die circadiane Uhr der Erbsenblattlaus.
Die Blattläuse haben auf Kurztagbedingungen mit einer niedrigeren Fruchtbarkeit und kürzerer Reproduktionsspanne reagiert. Kurze Tage haben jedoch nicht das Populationswachstum beeinflusst, da die Leistungseinbußen erst spät im Leben des Individuums in Erscheinung traten. Im Gegensatz zur Erbsenblattlaus entwickelte sich *E. balteatus* 13 % schneller unter Kurztagbedingungen und erbeutete signifikant mehr Blattläuse, während *C. carnea* sich 13% schneller unter Langtagbedingungen entwickelte und marginal höhere Prädationsraten erreichte. Diese Ergebnisse verdeutlichen, dass die Tageslänge wichtige Aspekte der Biologie von Organismen beeinflusst, aber dass die Richtung und Bedeutung von Art zu Art unterschiedlich ist.
Ich nahm an, dass die Einschränkungen oder Vorteile durch ein verkleinertes oder vergrößertes Zeitbudget bestimmt werden und daher von der zeitlichen Nische abhängen. *E. balteatus* ist tatsächlich nachtaktiv, während *C. carnea* dämmerungsaktiv zu sein scheint. Für *A. pisum* existieren hingegen nur unzureichende Daten. Daher züchtete ich *A. pisum* auf künstlichem Futter

und nahm Überlebensraten, Häutung und Honigtau-Exkretion auf. Die Aktivitätsmuster waren deutlich rhythmisch, und Häutung und Honigtau-Exkretion waren tagsüber erhöht. Daher kann die Einnischung auf Tagaktivität die beobachteten (aber schwachen) Nachteile kurzer Tage erklären.

Die zeitliche Nische einiger Organismen ist überraschend flexibel, und eine flexible zeitliche Nische könnte erklären warum der Effekt der Tageslänge relativ niedrig in *A. pisum* war. Daher untersuchte ich die circadiane Uhr der Erbsenblattlaus, da dieser Mechanismus die Aktivitätsmuster reguliert.

Zunächst verbesserte ich das künstliche Futter von *A. pisum*, und fügte den Lebensmittelfarbstoff Brilliant Blue FCF hinzu. Dieser Farbstoff färbte sowohl Magen als auch Honigtau in niedriger Konzentration ohne die Mortalität zu erhöhen, und machte dadurch die Exkretion von Honigtau unter schwachem Rotlicht sichtbar. Ich nutzte anschließend das blaue Futter, um Blattläuse einzeln in 16:08 LD und konstanter Dunkelheit (DD) aufzuziehen und dabei Honigtau-Exkretion und Häutungen alle drei Stunden zu notieren. Zusätzlichen nutzten wir ein neues Überwachungssystem um Aktivitätsmuster in Lokomotion kontinuierlich in LD und DD aufzuzeichnen. Sowohl Lokomotionsrhythmik als auch Honigtau-Exkretion von *A. pisum* schienen bimodal zu sein und erreichten früh morgens und nachmittags ihre Maximalwerte in LD. Beide Rhythmen bestanden auch unter konstanter Dunkelheit einige Zeit fort, was aufzeigt, dass die Rhythmen von einer funktionierenden inneren Uhr gesteuert werden. Die Rhythmik im Metabolismus dämpfte jedoch innerhalb von drei bis vier Tagen aus, während die Lokomotionsrhythmik mit einer komplexen Verteilung verschiedener free-running-Perioden fortbestand. Diese Ergebnisse passen zu einer gedämpften circadianen Uhr, die aus mehreren Oszillatorgruppen besteht. Ein solches Modell wurde vorgeschlagen, um circadiane Uhren mit Messungen der Photoperiode zu verknüpfen, aber nie empirisch überprüft.

Insgesamt verbinden meine Versuche die Einschränkungen phänologischer Anpassung mit einer mechanistischen Erklärung. Ich zeigte, dass kürzere Tage einigen Arten eines trophischen Netzwerks Vorteile, anderen jedoch Nachteile verschafften, und habe diese Unterschiede auf die zeitliche Nische der Arten zurückgeführt. Ich habe weiterhin gezeigt, dass eine flexible circadiane Uhr die Nachteile lindern kann, möglicherweise weil sie die Plastizität der zeitlichen Nische erhöht.

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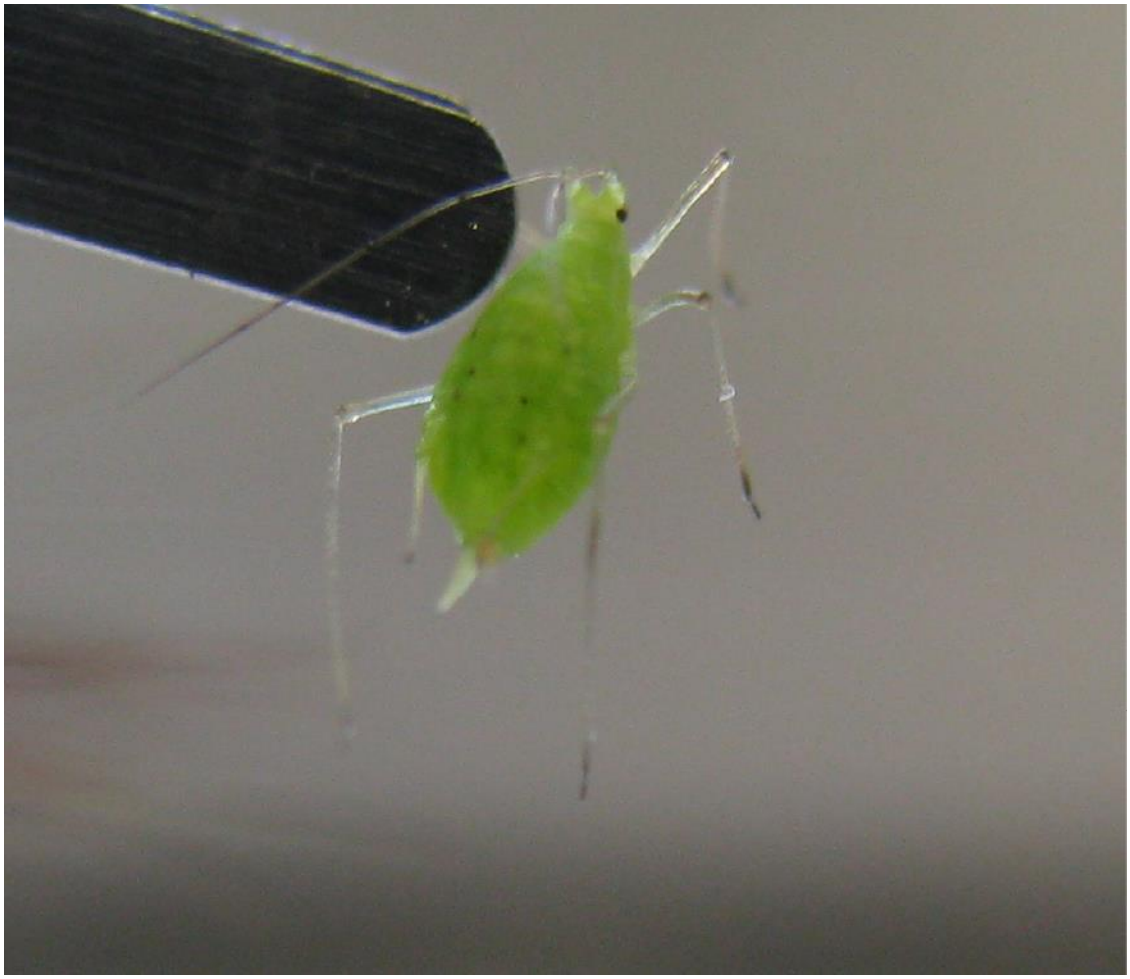


Acyrthosiphon pisum (Harris), the focal model organism in this thesis

CHAPTER I: GENERAL INTRODUCTION

Ecology analyses how organisms cope with their environment (Haeckel, 1866), and how they adapt to changes. One pervasive source of change is the day/night cycle, and there are numerous strategies to live with diurnal change (Moore-Ede et al., 1982). For example, many ectothermic species are diurnal (e.g. reptiles, Vitt & Zug, 2012), whereas endothermic mammals are often nocturnal (Gerkema et al., 2013). Other activity patterns include being active during dusk and dawn (Aschoff, 1966), relying on tidal and lunar rhythms (Tessmar-Raible et al., 2011), or abolishing rhythmicity altogether, as some species do at the poles (Blix, 2016) or during long-distance migration (Bloch et al., 2013). This diversity in timing affects ecology on all scales, from individual fitness to social interactions, species networks and biogeography - yet the role of rhythms in ecology is under-appreciated.

Diurnal rhythms are central to ecology

Diurnal rhythms have direct effects on individual fitness and well-being. For example, humans are diurnal animals (Roenneberg et al., 2003b), so physical and mental performance vary over the course of the day (Atkinson & Reilly, 1996). There are inherent advantages in structuring diurnal activity and in being able to anticipate change (Vaze & Sharma, 2013). Anticipation of day and night helps for example plants to maximize photosynthesis (Dodd et al., 2005), primes animal metabolism to feeding times (Bass, 2012), and provides migratory butterflies with a reference for their compass (Zhu et al., 2008). These examples show the range of benefits that diurnal rhythmicity provides to individuals.

Diurnal rhythmicity also affects interactions with others. For example, diurnal rhythms synchronize individuals in order to swamp predators (Santos et al., 2016), or desynchronize groups to avoid cannibalism (Pizzatto et al., 2008). The right timing has direct fitness consequences, e.g. birds that sleep longer obtain fewer extra-pair matings (Poesel et al., 2006) and instead suffer from cuckoldry themselves (Greives et al., 2015). Moreover, competing species can partition their niches in time, thus avoiding food competition (Mahendiran, 2016) or intra-guild predation (Ximenez-Embun et al., 2014). Mutualisms, on the other hand, require interacting species to tune their rhythmicity to the same time of day (Wier et al., 2010). In agonistic relationships such as parasitism, selection pressures differ, which may lead to an arms race around the clock (Martinez-Bakker & Helm, 2015). Hence, in addition to affecting individual health, diurnal rhythmicity regulates the strength of interactions both on the species level and among species.

Diurnal rhythms are driven by the circadian clock

Diurnal rhythms are regulated by the circadian clock. Circadian clocks are present throughout the tree of life, i.e., in archaea (Whitehead et al., 2009), bacteria (Golden, 2003), and eucaryotes like plants (Hsu & Harmer, 2014) and mammals (Reppert & Weaver, 2002). All clocks have in common that a molecular feedback loop causes an oscillation in protein levels with a period of circa 24 hours; the protein level can be used by other physiological processes as indicator of time of day. The core clockwork of *Drosophila melanogaster* consists of two interlocked negative feedback loops (Hardin, 2011): The heterodimer Clock/Cycle (CLK/CYC) activates the expression of the genes period (*per*) and timeless (*tim*). Accumulating PER and TIM proteins then inhibit CLK, thereby regulating their own expression. This mechanism is sufficient to cause a turnover in proteins of approximately 24 hours, but the cycle still has to be synchronized (entrained) with the environment. The blue-light photopigment Cryptochrome (CRY) is activated by light, and degrades TIM and PER upon activation, so that the clock is reset and synchronized with the day/night cycle. The combination of oscillation and entrainment can adapt an organism to the change of day and night.

Most circadian clock research in insects uses the model organism *Drosophila melanogaster* due to the availability of genetic tools (Peschel & Helfrich-Förster, 2011). But one cannot simulate the complexity of nature with the few model organisms currently in the laboratory (Kronfeld-Schor et al., 2013), and broad conclusions on clock mechanisms require testing organisms with varying phylogeny and life-history strategies (Bradshaw & Holzapfel, 2010). Thus our understanding of clock mechanisms does not represent the diversity of timing strategies.

Does the circadian clock affect the time budget?

While diurnal rhythmicity and circadian clocks are adaptive (Vaze & Sharma, 2013), a rigid temporal organization also has its drawbacks. The adaptation to the most beneficial temporal niche is generally associated with the evolution of specialized traits, which makes it difficult to reverse temporal patterns and may limit the niche breadth of the organism. For example, nocturnal owls cannot forage during daytime, even if this causes higher nestling mortality (Zarybnicka et al., 2012). Yet some species exhibit remarkable behavioral plasticity and are not constrained by their diurnal rhythms (Payne et al., 2013). It is tempting to conclude that differences in diurnal constraints are caused by different clock mechanisms, but literature about the flexibility of clock mechanisms to varying time budgets is surprisingly scarce. In particular, it is largely unknown how clock properties affect organisms in nature (Kronfeld-Schor & Dayan, 2003; Marques &

Waterhouse, 2004), as only few studies connect circadian clocks with evolutionary ecology (e.g. Helm & Visser, 2010). Without knowledge of the mechanisms it is difficult to predict the plasticity of a diurnal timing strategy.

Diurnal constraints can limit species distributions, because day length and thus the time budget vary with latitude. Feral goats on the Isle of Rum are limited in such a way by their strictly diurnal rhythm, and cannot expand to more northern latitudes with shorter winter days (Dunbar & Shi, 2013). On a global scale diurnal rhythms correlate with latitude and temperature (Bennie et al., 2014), and day length is one of the factors that contribute to a general diversity decline towards the poles (Anderson & Jetz, 2005). Overall, diurnal rhythms influence the use of day length, which limits the ecological niche to certain latitudes.

Phenology shifts and the use of day length

Global temperatures are rapidly rising since the last 100 years (Hansen et al., 2006; IPCC, 2014), and plants and animals of all taxa respond to changing conditions with phenology shifts (Parmesan & Yohe, 2003; Root et al., 2003). Although phenology shifts can re-establish a match of phenotype and temperature, they can disrupt links with other abiotic conditions and thus affect fitness (Zimova et al., 2016). For example, species that change their phenology are faced with novel day length – temperature correlations. Thus, seasonal organisms will have some life history stages exposed to novel day lengths. Similar to migration to different latitudes, the altered time budget may benefit or constrain the species, depending on the diurnal niche.

The cues to measure seasonal change likely differ among species (Visser et al., 2004), so there is no reason why phenology shifts should be equal across species. For example, one species may predict seasonal change by measuring mean temperatures, whereas another species relies on day length as cue. Indeed, different taxa and different trophic levels vary in their sensitivity to phenology shifts (Thackeray et al., 2016), and the resulting phenological mismatch can cause costs. For example, one study showed that plant phenology and caterpillar peak abundance have advanced, but that egg laying dates of great tits remained constant, so the birds suffered fitness costs from being out of synchrony with their prey (Visser et al., 1998). To understand the effects of phenology shifts on a trophic network, it is thus important to quantify the effects of phenology shifts separately for each species.

The collaborative research center "Insect timing"

Similar to the role of the clock in diurnal plasticity, integrative studies on the physiological basis of phenology change are scarce (Visser et al., 2010). To foster collaborations across disciplines, a collaborative research centre (CRC 1047 "Insect timing: mechanisms, plasticity and interactions) was founded in 2013 at the University of Würzburg. The CRC is a DFG – funded joint research project by ten departments from different sub-disciplines, including molecular biology, behavior and ecology. Close collaborations were formed within the CRC to integrate studies from different scales, from diurnal to seasonal timing, and from basic physiology to trophic interactions. In addition to the model system *Drosophila melanogaster*, research within the CRC encompassed a variety of non-model organisms for a comparative view on the impact of clocks in nature. As part of the CRC, I studied the effects of day length on life-history traits in a multi-trophic system of aphids and their predators. I then collaborated with the Department of Neurobiology and Genetics to focus on diurnality and the circadian clock as likely mechanisms for phenological constraints in the pea aphid.

Study organisms

While aphids are not as widely used as *D. melanogaster*, they are increasingly popular in studies of ecology and evolution (Brisson & Stern, 2006; Huang & Qiao, 2014), in part for their complex life cycle that consist of parthenogenesis during summer, but sexual reproduction in autumn (Simon et al., 2002). Aphids are distributed world-wide with about 4700 species, and placed together with sap-sucking Adelgidae and Phylloxeridae in the superfamily Aphidoidea, and with other phytophagous insect taxa into the Sternorrhyncha as suborder of the Hemiptera (bugs) (van Emden & Harrington, 2007). The pea aphid, *Acyrthosiphon pisum*, is particularly well-studied (Brisson & Stern, 2006). It is easy to rear, reproduces quickly, is relatively large, and it has been sequenced and annotated (The International Aphid Genomics Consortium, 2010).

Aphids are also interesting study organisms, because there is long tradition in researching the interaction of circadian clock and aphid phenology, which connects my two research questions. The seasonal switch from parthenogenetic to sexual reproduction is induced by shortening day length (Marcovitch, 1923). While day length measurements were generally thought to depend on the circadian clock (Bünning, 1936), aphids were among the first examples that appeared to be independent of the clock (Lees, 1973). A model with damping clock involvement has been proposed for aphids (Hardie & Vaz Nunes, 2001), and such a model could unite the differing views on circadian clock involvement in phenology (Saunders, 2005). Due to the historical importance of

aphids in linking the circadian clock with seasonal rhythms, I chose the pea aphid as focal organism.

Red form of *Acyrthosiphon pisum* (Harris)

Aims and main findings

To evaluate the constraints of differing time budgets, I tested the effects of day length individually on life history traits of the aphid *Acyrthosiphon pisum* (Harris, 1776) (Chapter III) and on two specialized predators, *Episyrphus balteatus* (De Geer, 1776) and *Chrysoperla carnea* (Stephens, 1836) (Chapter IV). I found that short day length constrained aphid fecundity and reproduction, although it did not result in lower population growth. The two aphid predators were more strongly affected by day length, and the direction of the effect matched to their diurnal niches. I then studied the diurnal rhythm of *A. pisum* on an artificial diet, hypothesizing that the short day constraints are caused by diurnality (Chapter V). As expected, aphids were day-active and the rhythmicity was independent of the host plant.

To link the fitness constraints to a mechanistic basis, I planned to study the circadian clock of *A. pisum*. Methodological problems, including low visibility of the honeydew, precluded however further work in constant darkness. I thus refined the artificial diet and used the colorant Brilliant Blue FCF to obtain visibly colored honeydew (Chapter VI). With the help of the refined diet, I then studied the aphid clock in a joint research project with the Department of Neurobiology and Genetics (Chapter VII). We showed that the aphid circadian clock is a weak oscillator that damps quickly, which explains why aphids were seen as hour glass models.

Aphid food (*Vicia faba*) in various growth stages.

CHAPTER II: GENERAL METHODS

All methods are described in chapters III-VII.

An individually bagged *Pisum sativum* plant with a small population of *Acyrthosiphon pisum.*

CHAPTER III: DAY LENGTH CONSTRAINTS OF APHIDS

This chapter is published as:

Joschinski J, Hovestadt T, and Krauss J. 2015. Coping with shorter days: do phenology shifts constrain aphid fitness? *PeerJ* **3**:e1103.

ABSTRACT

Climate change can alter the phenology of organisms. It may thus lead seasonal organisms to face different day lengths than in the past, and the fitness consequences of these changes are as yet unclear.

To study such effects we used the pea aphid *Acyrthosiphon pisum* as model organism as it has obligately asexual clones, which can be used to study day length effects without eliciting a seasonal response. We recorded life-history traits under short and long days both with two realistic temperature cycles with means differing by 2°C. In addition we measured the population growth of aphids on their host plant *Pisum sativum*.

We show that short days reduce fecundity and the length of the reproductive period of aphids. Nevertheless this does not translate to differences at the population level, because the observed fitness costs only become apparent late in the individual's life. As expected, warm temperature shortens the development time by 0.7 days/°C, leading to faster generation times. We found no interaction of temperature and day length. We conclude that day length changes cause only relatively mild costs, which may not decelerate the increase in pest status due to climate change.

INTRODUCTION

Nearly all organisms need to cope with environmental heterogeneity and fluctuation; showing a plastic response in the face of such heterogeneity can be beneficial. For example, several species from the *Daphnia* complex (Cladocera) can grow a 'crown of thorns' in response to predator pressure (Petrusek et al., 2009), and *Daphnia magna* allocates variable amounts of energy to size and shape as adaptive induced response to predator presence (Rabus & Laforsch, 2011). Similarly, many plants increase their investment into defence when attacked by herbivores (e.g. Agrawal, 2011). These examples demonstrate how phenotypic plasticity can affect fitness. One of the most important fitness traits is phenology (Chuine, 2010; Helm et al., 2013), i.e., the timing of life cycle events. Plasticity in phenology can profoundly change the ecology of a species, as it can alter the timing of critical life-history events and synchrony with other trophic levels (Visser et al., 1998; Visser & Holleman, 2001). Thus phenological plasticity is an important component of the ecology and evolution of species.

Phenotypic plasticity can not only be adaptive in temporally fluctuating environments, but also prevent extinction in environments under directional change (Chevin et al., 2013). The current rate of environmental change is likely unprecedented since 1400 years (IPCC, 2013), as the global surface temperature rises by 0.2°C per decade (Hansen et al., 2006). Climate change modifies the onset and duration of seasons (Räisänen & Eklund, 2012), and many species have already responded by shifting their phenology in the according direction (Rosenzweig et al., 2007). By adjusting phenology via plastic responses, organisms can possibly mitigate the extinction risk imposed by climate change (Charmantier et al., 2008; Vedder et al., 2013), and even profit from it (Bell et al., 2015).

However, the evolution of phenotypic plasticity may be constrained by costs and limits (DeWitt et al., 1998). For example, plasticity can be limited by tightly interacting species, which may not shift their timing in synchrony. Among the best studied examples are great tit populations which have lost synchrony with their caterpillar prey (Visser et al., 1998), and winter moths which are no longer synchronous with their host (Visser & Holleman, 2001). We hypothesize that another limit of plasticity is posed by the reduction in day length (photoperiod) associated with a shift in phenology: First, activities of a diurnal species, e.g. foraging, can be constrained by shorter days, if individuals live in a later time of the year. Secondly, photoperiod is the most common cue to predict seasonal change (Saunders, 2013). Photoperiodism is commonly assumed to be based on the circadian clock (Bünning, 1936; Saunders, 2013), a molecular clockwork which governs rhythmicity (Peschel & Helfrich-Förster, 2011). Thus we hypothesize that altered day length conditions interfere with the (yet unresolved) interplay of seasonal and circadian rhythmicity and hence affect phenotypic plasticity.

The effect of warming temperature on fitness is relatively well established. Within physiological limits warmer temperature generally speeds up metabolic rates (Gillooly et al., 2001). Less researched, and potentially important in a changing climate, are interactions of day length and temperature. We propose that warmer temperature results in faster growth during the organism's active period, but higher energy expenditure during resting time. Hence, the effect of temperature should depend on day length. Also, temperature might enhance the interference with circadian timing, as the clockwork is not fully compensated for temperature changes (Saunders, 2014). Thus, short day conditions may decrease insect fitness, whereas warm temperature should enhance growth rates, and warming might enhance the fitness costs of short days.

Aphids like *Acyrthosiphon pisum* (Harris) are well suited to study constraints of short days. During summer *A. pisum* reproduces clonally, establishing exponentially growing populations. Live-born nymphs have, however, little chance to survive sub-zero temperatures (Simon et al., 2002). Therefore, in many clones aphids give birth to a single generation of sexual morphs in autumn, which produce cold-resistant eggs to overwinter. In warmer climates this response to photoperiod is frequently lost, so asexual aphid morphs are active throughout the year (Simon et al., 2002). These differences in phenology within one species allow studying day length effects in a seasonal insect without actually inducing a photoperiodic response.

Specifically we hypothesize, that

(1) Shorter day length constrains aphid performance and reduces population growth.

(2) Warm temperature causes quicker generation cycles and faster population growth.

(3) Temperature and day length interact, so that the positive effects of an increase in ambient temperature decline with shorter day length.

We therefore expect fitness costs under short-day conditions compared with long-day conditions, and possibly the lowest fitness under short days combined with warm conditions.

MATERIALS AND METHODS

To test for constraints of phenotypic plasticity, we carried out experiments with an asexual clone of the aphid *A. pisum* in four climate chambers at the individual as well as at the population level. We measured population growth on whole plants of *Pisum sativum* (L.), and life history data of individuals raised on cut leaves of *P. sativum*.

Day length and temperature settings

We used four identical climate chambers (Sanyo/Panasonic MLR-H series), in which we applied two realistic temperature settings with sinusoid day/night cycles, ranging from 12-23°C (±1°C) and from 14-25°C (±1°C), and two day length regimes with day length of 12:12 LD and 16:8 LD (Fig. III.1), using 40W fluorescent lamps. The temperature differed between the light treatments at dawn and dusk, but this difference in light sums is only 1.2 %. Treatments were exchanged weekly, because the maximum light intensities varied between chambers from 13,000 – 21,000 lux. Because development and reproductive period lasted four weeks, all treatments received the same light sum [lux * h] over this period. The lower temperature settings in the experiment approximately reflect naturally occurring temperatures in Würzburg, southern Germany, during summer solstice (12-22°C) and during beginning of September (11-22°C; data from Deutscher Wetterdienst, http://www.dwd.de/). The higher temperature settings simulate climate change with moderately increased mean temperature of 2°C, which ranges between the SRES B1 and B2 marker scenario projections for 2099 (IPCC, 2000). We are aware that this is a conservative estimate; we nevertheless used this low difference of means, so that we did not confound the results by exceeding the physiological optimum of the pea aphid.

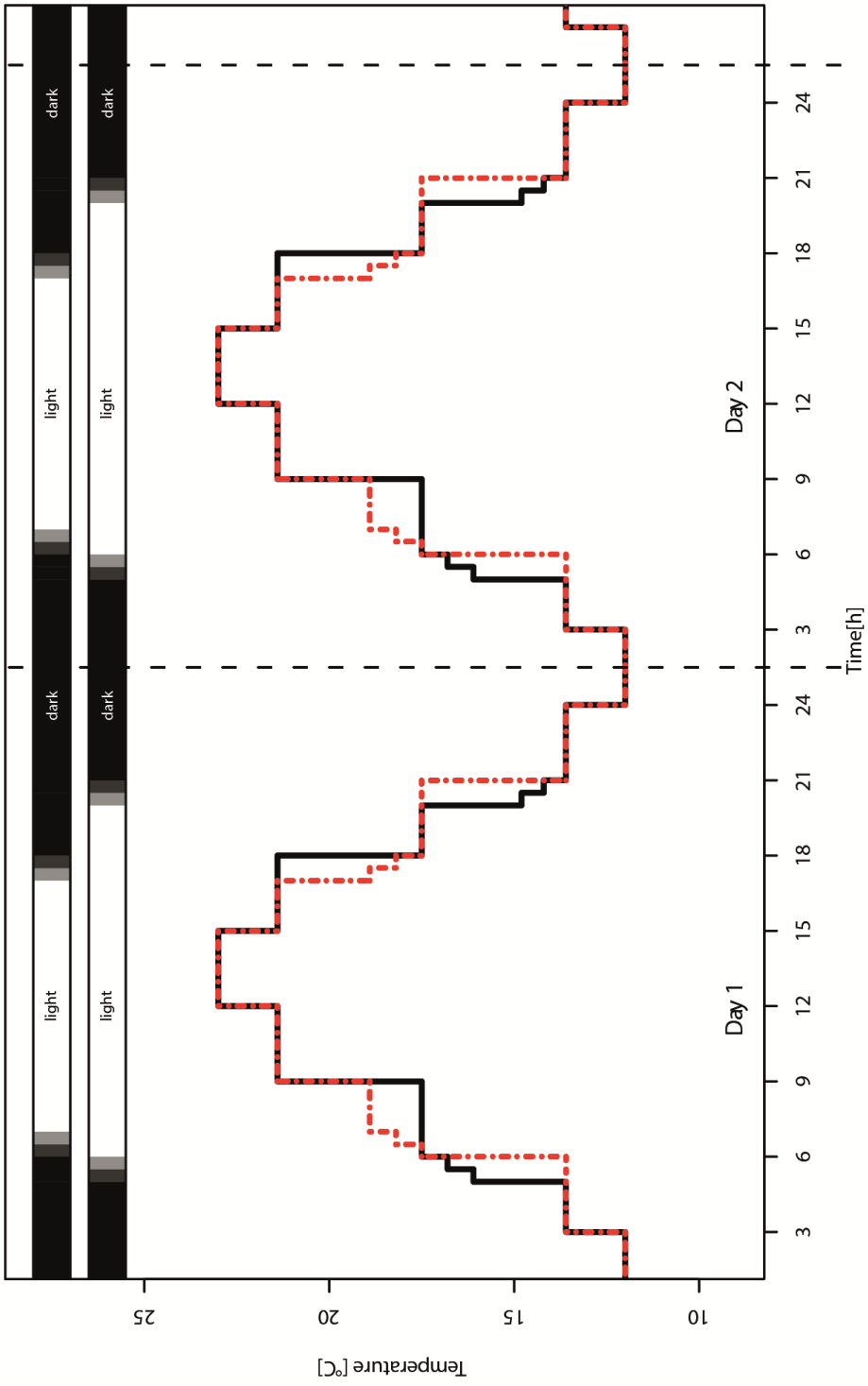


Fig. III.1: Warmtemperature settings for long day (solid lines, lower bar) and short day (dashed lines, upper bar) treatments. Mean temperatures of long and short day conditions do not differ. The temperature in the two low temperature treatments was overall 2°C lower (not shown).

Study organisms

Due to its fast population growth and its properties as virus vector, *Acyrthosiphon pisu*m (Harris, Aphididae) is a pest in agriculture, which is distributed throughout northern Europe, North America and New Zealand (Blackman & Eastop, 2000). *Acyrthosiphon pisum* feeds on legume crops such as pea (*Pisum sativum* L.) and bean (*Vicia faba* L.), and does not switch hosts in autumn. The aphid clone L1_22, an asexual green alfalfa biotype, was kindly provided by Grit Kunert (MPI Jena). The known asexuality of the clone has been confirmed by providing an 8:16 LD rhythm at 10°C for four generations.

*Pisum sativ*um L. is a suitable host plant for *A. pisum*, and agricultural plants are frequently attacked by aphids (Blackman & Eastop, 2000). We used the breed 'Kleine Rheinländerin' (Bingenheimer Saatgut, Echzell, Germany), which grows to 40 cm, for all experiments.

Performance of individual aphids

To detect day length and temperature effects on the individual performance of aphids, we placed 20 adult apterous, asexual aphids per climate chamber (20 x 4 = 80) singly in plastic tubes (8 x 3.5 cm), and used their first born nymphs (termed first generation) as new focal individuals for further measurement. These first-generation nymphs were fed every second day with one cut leaf each, and we recorded development time, length of reproductive period, post-reproductive period and life span. We used cut leaves like Meister et al. (2006) to exclude differences in food quality, as a living host plant can be expected to fix more carbon under long day conditions. We counted and discarded newly born nymphs daily (thus measuring daily fecundity and lifetime reproductive output of each focal animal). In order to test for maternal effects in a second generation and to confirm the loss of sexuality in the clone we retained one early-born nymph per focal aphid after 11-13 days. Additionally, we retained one late-born nymph after 29-31 days, because we expected the maternal effects to intensify as the adult ages. We raised all aphids of the second generation under the same conditions (16:8 LD, 22°C and 60% humidity), so that maternal effects could be distinguished from direct effects of day length and temperature. These second generation aphids were fed with fresh plant material every second day, and life history parameters were also recorded every second day.

To supply the aphid individuals with food we grew 60 pea plants ('Kleine Rheinländerin') per week with two plants per pot (11x11 cm, filled with Einheitserde® classic soil, Einheitserdewerk Hameln GmbH, Sinntal, Germany) over six weeks at 22°C, 16:8 LD and 60% humidity, so that 2-3 week old plant material (approximate BBCH growth stage 14-15) was available over the whole

course of the experiment. Pea plants grow pinnate compound leaves with morphologically different stipules. We fed four leaflets from the same leaf compound (the youngest which had completely unfolded leaflets), but excluded the basal stipulate leaves. If there was not enough plant material available, we fed the aphids with plant material of two leaf compounds of similar age. The four leaflets were randomly distributed over the four treatments to ensure that all treatments received the same plant quality. We used the same plant no more than twice in order to avoid induction of defense. The plants were always raised at 22°C and in a 16:8 LD cycle.

Altogether 80 individuals of the aphid *A. pisum* were used in the experiment. Nine aphids died before reaching reproductive age, and six individuals (7.5%; five under cold, short day and one under warm, short day treatment) developed into alate (winged) virginoparous morphs. The 15 deceased or winged individuals were excluded from further analysis. A further ten aphid individuals were accidentally killed as adults, which reduced the number of replicates to 55 aphids for the traits fecundity, reproductive period, post-reproductive period and life span.

Population experiment

To detect the effects of day length and temperature on population demography, we sowed 60 pea plants into 11x11cm square pots filled with a peat-based substrate (Einheitserde® classic, Einheitserdewerk Hameln GmbH, Sinntal, Germany). The plants were watered from above during the first week and from below (using felt mats) thereafter in four trays with 15 plants each. We kept all plants in a walk-in climate chamber with 22°C at 16:8 LD and 60% humidity and watered five times per week. After 18 days we fixed each plant with raffia fibres to 50 cm wood sticks. After 25 days 12 plants from each tray were evenly distributed over the four climate regimes (48 plants in total), and the position within each chamber fully randomized. Following one week of acclimation we established aphid populations by placing 10 individuals of adult apterous (wingless) asexual morphs on each individually bagged plant, using micro-perforated plastic bags (255x700 mm, 0.5 mm perforations, Baumann Saatzuchtbedarf, www.baumann-saatzuchtbedarf.de). To accommodate for climate chamber differences we exchanged treatments between chambers weekly. We estimated population size weekly by counts of alate (winged) and apterous adults and nymphs (judged by the visibility of the cauda and size differences) over the course of four weeks on the living plants (BBCH growth stages approximately 16-19). To control the effect of heat stress on the plants we distributed 24 aphid-free, 23 days old plants over the four chambers to observe plant responses to the artificial climate over four weeks.

Statistics

We used R version 2.15.2 (R Core Team, 2012) for all analyses. On the individual level, 65 out of 80 aphids were used to assess development time, and 55 for the remaining variables (length of reproductive period, length of post-reproductive period, life span and fecundity). We tested effects of day length, temperature and their interactions as main factors in two-way ANOVAs on all of those parameters except fecundity. The latter we used to construct a Leslie Matrix to yield the theoretical population rate of increase r_t and the reproductive values of each age cohort (Leslie, 1945). We used a Leslie matrix, because averaged daily fecundity (as for example used by Meister et al., 2006) does not account for skews in the fecundity curve, which cause shorter generation times and alter growth rate projections. In particular, late-born offspring add very little to population growth compared to early-born offspring, and the true fitness costs may be over- or underestimated. We used the estimates of r_t in a two-way ANOVA to also test for effects of day length and temperature. At the population level, we calculated the weekly population growth rates r_1 ,r_2 and r_3 on 48 plants, as $(N_x/N_x\text{-}1)$, using the aphid number N at week x, and the daily growth as $r_x\text{^}(^1/_7)$. We compared the rates of increase, i.e. log (growth rates), in two-way ANOVAs as before. Because a temperature gradient existed within the climate chambers, the position within chambers had a significant effect for nymphal development and r_t. However, as the position effect was in the same direction as the effect of temperature and did not qualitatively change the results, we omitted it from analysis.

RESULTS

Life history traits of individual aphids

In our experiment aphids developed on average within 10.7±0.2 days and warm temperature shortened the development time significantly (Fig. III.2, Table III.1, Table III.2). The length of the reproductive period (Fig. III.2, Table III.1) and the fecundity of aphids (Fig. III.3, Table III.1) depended solely on day length. Aphids raised under short-day conditions reproduced about 3 days (14%) less, and produced 22% fewer nymphs (Table III.2). The post-reproductive period ranged from 5.0±0.6 (warm, long) to 9.8±1.3 (cold, short) days, and was elongated by a reduction of day length and of temperature (Fig. III.2). Overall, warm temperature shortened the total life span, i.e. the sum of development time, reproductive and post-reproductive period. Even though the food quality was sufficient for full development (including the post-reproductive period) of all focal aphids in the first generation, the second generation suffered high mortality rates (34%) and reduced offspring numbers (to 0-30%). 73 out of 75 surviving adults of the second generation

reproduced and no males were observed; so we confirm that the focal aphids did not switch from asexual to sexual offspring. The theoretical population rates of increase r_t (based on Leslie matrices) differed significantly between temperature regimes, but were independent of day length (Fig. III.4a). The reproductive values of the last three days of reproduction were on average 1.56, which is 9.7% of the maximum reproductive value (16.99). The average growth rate was below the growth rate of the population experiment (see next section), possibly because the cut leaves do not provide enough phloem pressure.

Fitness costs on the population level

In the population experiment, about 10% of the observed aphids were adults, and 0% (in the first two weeks) to 13% (in the third week) of the adults were winged. Adult/nymph ratios and winged/wingless ratios never varied significantly among treatments (all $p>0.1$), so differences in wing induction patterns are unlikely to have affected our results. Aphid density (sum of nymphs, winged and wingless aphids) increased exponentially over the first two weeks, with a weekly growth of about one order of magnitude (Fig. III.4b). After two weeks aphid densities were higher in the warm treatment (1027±101 aphids) than in the cold treatment (668±42 aphids), but not significantly affected by day length (Table III.1). In week three the exponential growth ceased, and during weeks three and four most plants died and aphid densities declined, especially in the warmer treatments. Control plants without aphids did not show any signs of heat stress and were healthy throughout the experiment.

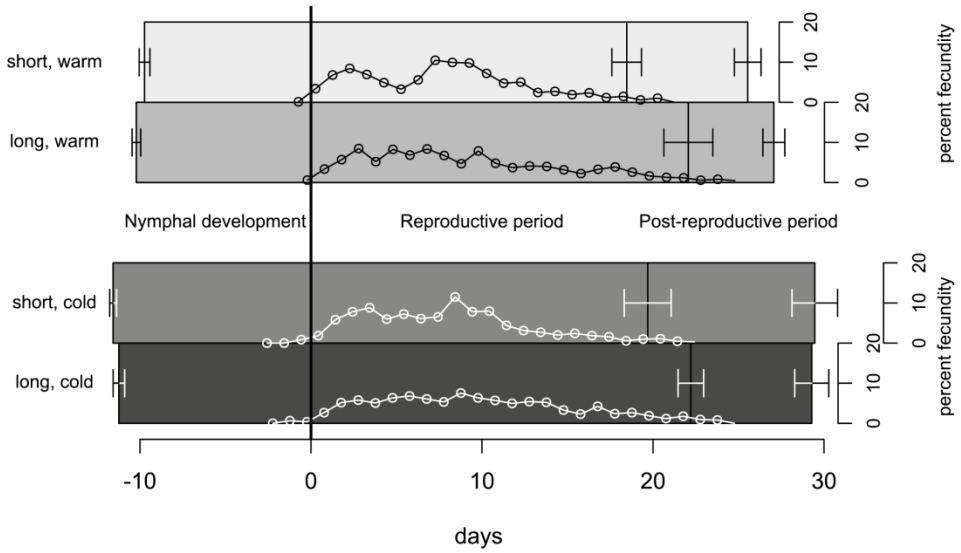


Fig. III.2: Life-history traits of individuals reared under different climate conditions. The bars are aligned at the mean onset of reproduction (i.e. not left-aligned) to better distinguish temperature effects (on development) from day length effects (on reproduction). Bars indicate S.E. Lines with open circles indicate the timing of nymph production (expressed as daily contributions to total fecundity in %). These curves form also the basis for the Leslie calculations (Table III.1, Table III.2). Statistics see Table III.1.

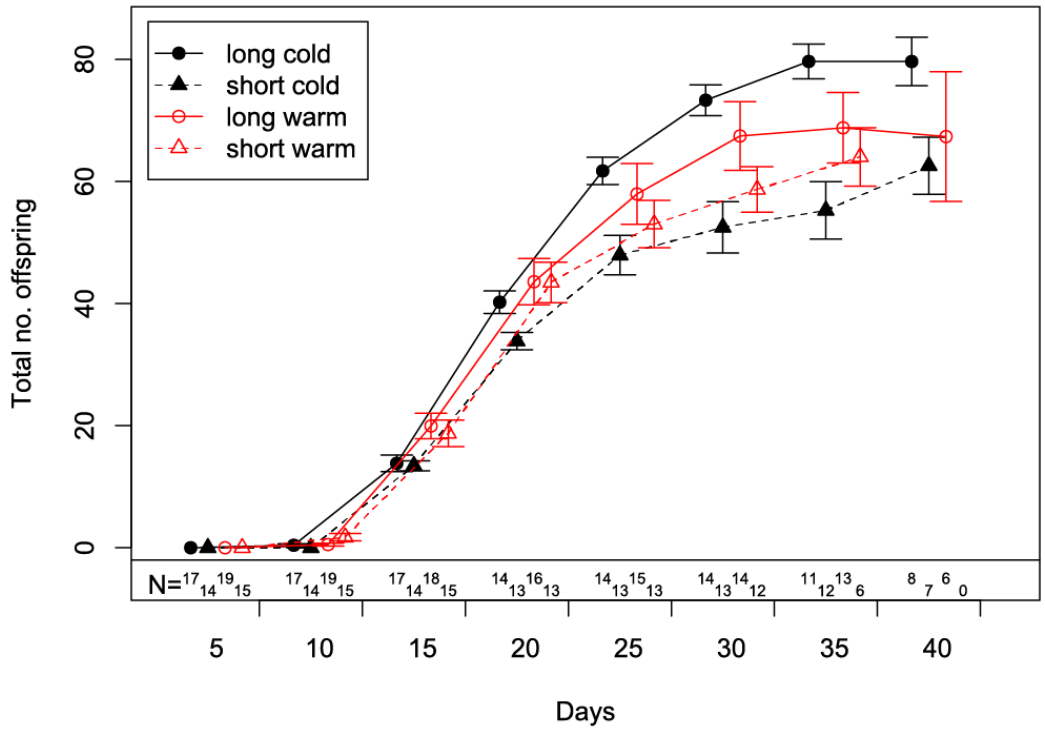


Fig. III.3: Cumulative fecundity as function of age of individuals reared under four climate conditions. Bars indicate S.E. Statistics see Table III.1. Sample size (N) declines over time, because the aphids die of age (c.f. Fig. 2).

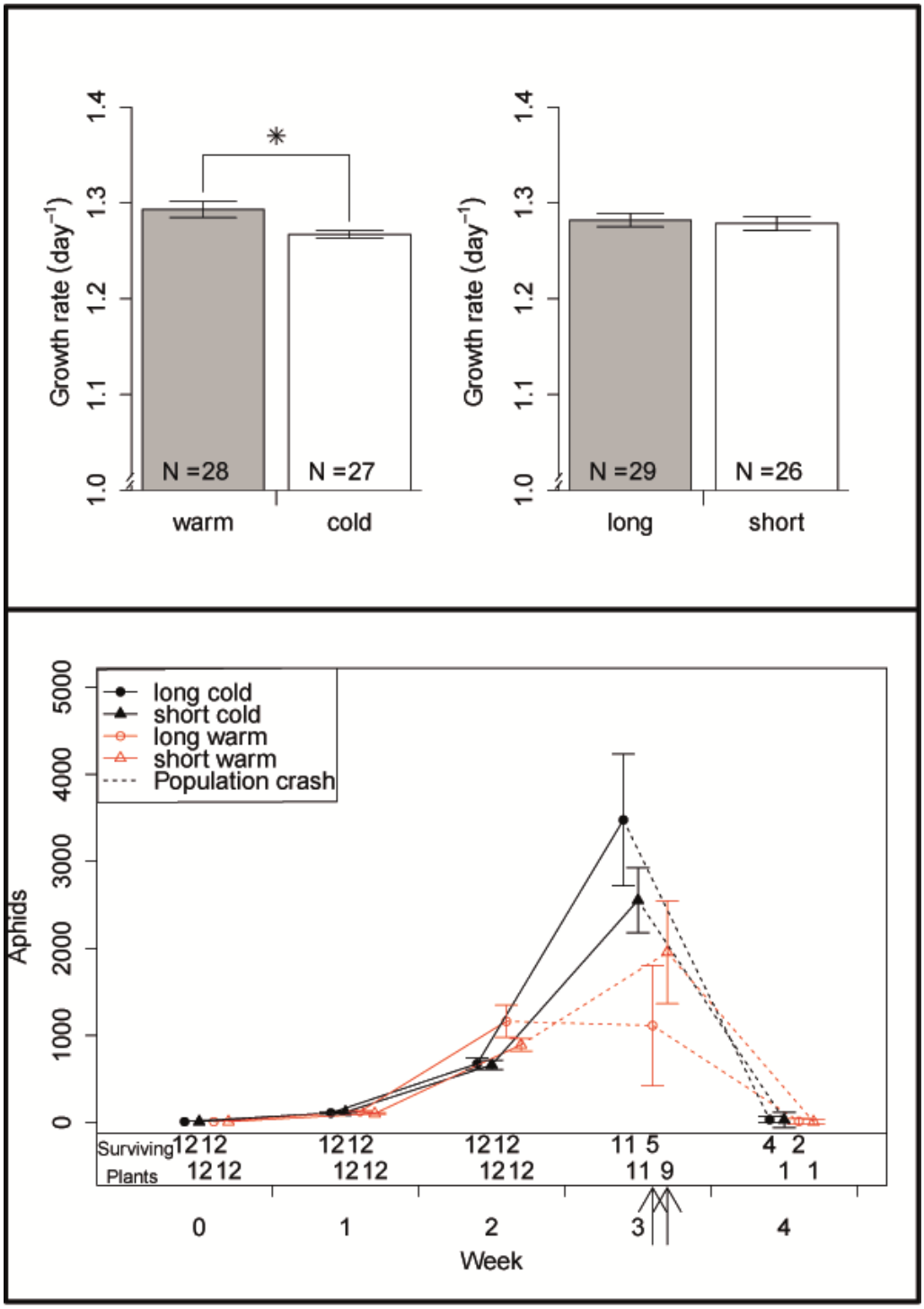


Fig. III.4: Growth rates of aphids under warm and cold conditions at 16:8h and 12:12h day length. Statistics see Table III.1. a) Comparison of population growth under warm vs. cold and under long day vs. short day conditions. Data is based on Leslie-matrices derived from individual life histories. Bars indicate S.E. b) Population growth of aphids reared on whole plants. In week 3 the exponential growth ceased, and in weeks 3 and 4 the populations crashed (dashed lines; indicated by arrows).

Table III.1 ANOVA tables testing for day length and temperature effects on aphid life history traits. Significant effects are shown in bold.

Response variable	Factor	F	df	p (<F)
Development time	Temperature	23.62	3,64	**<0.001**
	Day length	0.10	3,64	0.759
	Temp x day length	2.01	3,64	0.162
Reproductive period	Temperature	0.27	3,54	0.603
	Day length	6.98	3,54	**0.011**
	Temp x day length	0.22	3,54	0.643
Post-reproductive period	Temperature	6.36	3,54	**0.015**
	Day length	6.22	3,54	**0.016**
	Temp x day length	0.11	3,54	0.747
Life span	Temperature	9.24	3,54	**0.004**
	Day length	0.33	3,54	0.567
	Temp x day length	1.22	3,54	0.274
Total fecundity	Temperature	1.33	3,54	0.253
	Day length	12.84	3,54	**<0.001**
	Temp x day length	2.70	3,54	0.107
R_t (rate of increase derived from life-history traits)	Temperature	6.90	3,54	**0.011**
	Day length	0.08	3,54	0.773
	Temp x day length	2.95	3,54	0.092
Population rate of increase	Temperature	4.92	3,41	**0.032**
	Day length	0.04	3,41	0.836
	Temp x day length	0.54	3,41	0.465

Table III.2. Effect sizes of the four day length/temperature treatments on aphid life history traits.

Response variable		**Short day**		**Long day**	
Development time [days]	Low temp	11.6	(±0.2)	11.2	(±0.3)
	High temp	9.7	(±0.3)	10.2	(±0.3)
Reproductive period [days]	Low temp	19.7	(±1.4)	22.2	(±0.8)
	High temp	18.5	(±0.9)	22.1	(±1.4)
Postreproductive period [days]	Low temp	9.8	(±1.3)	7.1	(±1.0)
	High temp	7.1	(±0.8)	5.0	(±0.6)
Life span [days]	Low temp	41.1	(±1.2)	40.3	(±1.4)
	High temp	35.0	(±1.1)	37.4	(±1.8)
Total fecundity [nymphs]	Low temp	54.2	(±4.7)	77.7	(±2.9)
	High temp	56.5	(±4.1)	65.3	(±5.5)
R_t (rate of increase	Low temp	0.23	(±0.003)	0.24	(±0.004)
derived from life- history traits)	High temp	0.26	(±0.009)	0.25	(±0.010)
Population rate of increase	Low temp	0.24	(±0.010)	0.25	(±0.017)
	High temp	0.31	(±0.012)	0.29	(±0.039)

DISCUSSION

Plasticity in phenology likely helps to make use of novel climate conditions and to extend the asexual season, which may increase the pest status of aphids (Bell et al., 2015). However, the novel day length conditions under which the animals live may be non-optimal to the organism, and thus reduce the advantage of plasticity. Our results show that a 2°C increase in temperature accelerates development and increases the population growth in an asexual aphid clone, but does not alter the individual reproductive period or fecundity. In contrast to increased temperature a shorter day length reduced the length of the reproductive period by 14% and fecundity by 22%, but did not significantly affect development time or life span.

Day Length

In our experiment day length alters fecundity and length of the reproductive period and aphids suffer under short- day environments from reduced reproduction.

Even though variation in phenological traits is commonly regarded as phenotypic plasticity (Charmantier et al., 2008; Vitasse et al., 2010; Vedder et al., 2013), the microevolutionary costs and limits of plasticity (sensu DeWitt et al., 1998) in phenology have to our knowledge never been measured. Phenotypic plasticity in phenology often relies on day length (photoperiod) as cue, and our study is the first that demonstrates fitness costs linked to short days in insects. On living plants aphids exhibit circadian rhythmicity and seem to be day-active (Eisenbach & Mittler, 1980; Hodgson & Lane, 1981; Cortes et al., 2010), which offers – in agreement with the hypothesis outlined in the introduction – a tentative explanation for the observed fitness loss under short days. Further studies will need to verify the diurnality independent of host plants, and to measure phloem consumption under long and short days.

Photoperiod may also have a less direct effect on fitness, as its measurement may be based on the circadian clock (Bünning, 1936), an endogenous time-keeping mechanism which relies on two cyclically expressed protein complexes, PERIOD/TIMELESS and CLOCK/CYCLE ((Peschel & Helfrich-Förster, 2011). Interference among seasonal rhythm and circadian clock seems reasonable, though this hypothesis is still under debate (Danks, 2005; Kostal, 2011). Hence shortening day length may not only affect the time available, but also its correct measurement. So far, relatively little is known about the circadian rhythm of aphids, but with the recent identification of the clock genes in aphids (Cortes et al., 2010), further progress can be expected.

On the population level we did not detect effects of day length on fitness. Our calculation based on Leslie matrices indicates that short day length does not significantly dampen population growth,

because the additional offspring produced under long days are born rather late in the adults' life (c.f. Fig. III.2); thus only life stages with little reproductive value are affected. Consequently, substantial costs of shortened day length are not observed in our population experiment. We thus conclude that the observed reduced reproduction does not impede population growth.

Temperature

As expected, we found that warmer temperature shortens the life cycle of aphids. Because the quicker life cycle leads to faster population growth both in our Leslie calculations and on real plants, climate change with increased mean temperatures should increase the pest potential of aphids (Bell et al., 2015). Presumably warmer temperature acts on metabolic rates, as is well established for insects (Gillooly et al., 2001). Temperature did, however, not change fecundity or the length of the reproductive period over the measured range, and thus warm temperature per se does not affect an individual's condition. This contradicts studies of temperature on the condition of *A. pisum* by Campbell & Mackauer (1977) and Kaakeh & Dutcher (1993), but supports the results of Kilian & Nielson (1971). On a different aphid species, Rispe et al. (1996) also detected no general effect of temperature on fecundity, but large variation among clones. Clonal variation also explains differences between the cited experiments.

Because variability in temperature will likely increase due to climate change (IPCC, 2007), we included diurnal cycles in our design. Due to the nonlinear shape of the growth rate curve, variability should increase the growth rate as long as it is below the optimum (Estay et al., 2013). Several studies on other clones indicate that the physiological optimum of *A.pisum* lies beyond 20°C, and decreases only at temperatures higher than 25°C to 30°C (Kenten, 1955; Kilian & Nielson, 1971; Campbell & Mackauer, 1977; Kaakeh & Dutcher, 1993; Rispe et al., 1996). Our treatments lie with 17.5 and 19.5°C below the reported optimum, so one would expect a larger effect of an increase in mean temperature on reproductive traits in our experiment compared to experiments applying constant temperatures. However, this hypothesis was not supported by our experiment. Kilian & Nielson (1971) and Kaakeh & Dutcher (1993) recorded with constant temperatures around similar means (15/20°C) a shortening of development time by 0.7 and 1.2 days/°C, respectively. These values are largely in line with those in our experiment, where the onset of reproduction shifted by 0.7 days/°C. We found however some effect of temperature variability on longevity, because in contrast to Kilian & Nielson (1971), in our study life span decreased under long days by 1.4 days/°C. Possibly, our clone is adapted to colder temperature, so that the maximum temperatures of 25°C stressed the aphids and caused a hazard. Therefore higher temperature variability may decrease, not increase, aphid performance.

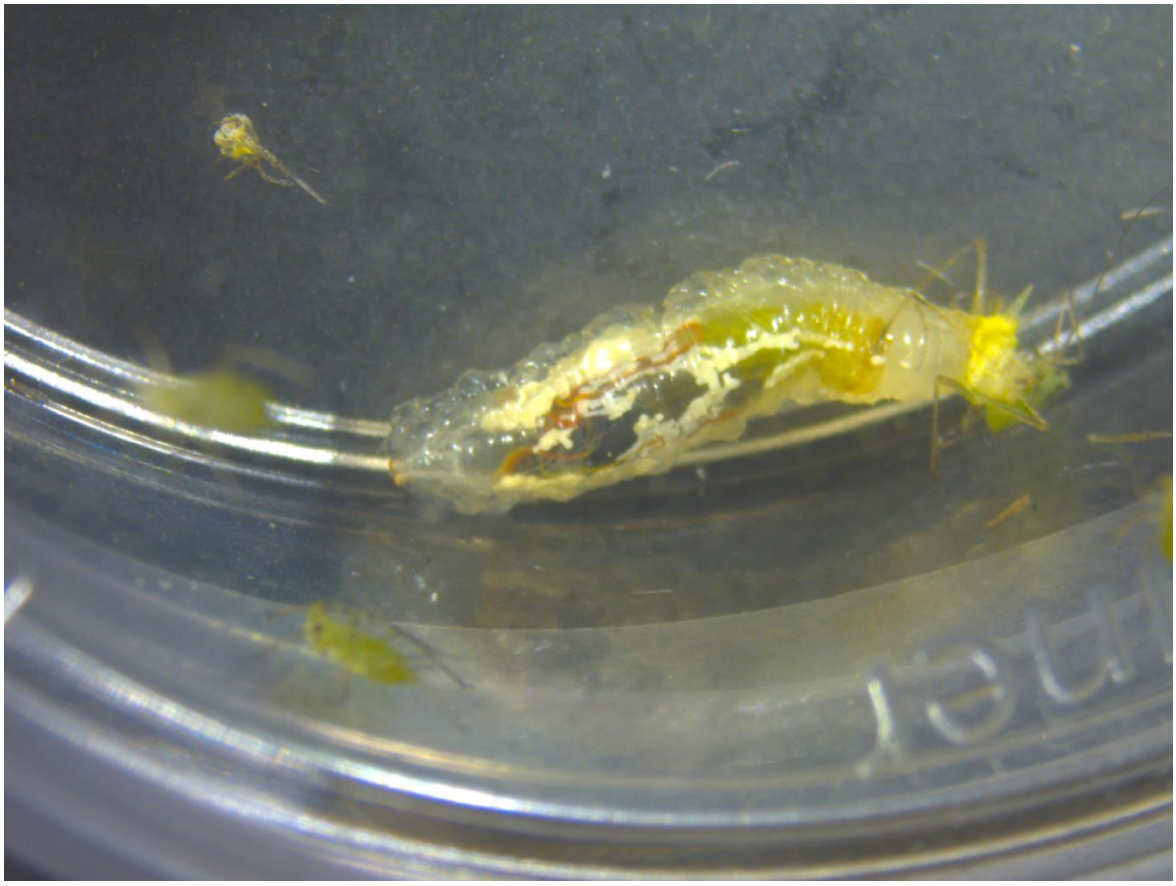
Contrary to our hypothesis that temperature has opposing effects at day and night, we found no interaction of day length and temperature. We hence conclude that day- and night time temperatures have similar effects on aphid fitness and impose physiological constraints only by generally affecting the aphid metabolism.

CONCLUSION

We show that a shorter photoperiod reduces reproduction in obligately asexual aphids. Consequently, the aphids' potential benefits following from global change are reduced, as temperature increase may lead to novel day length-temperature correlations. If the fitness decline has its roots in physiological constraints, our results may be extrapolated to any day-active insect species. However, these side-effects of phenotypic plasticity were not detected at the population level, because they affect only late fitness components in the individual's life. We further show that warm temperatures increase aphid growth by shortening development, but neither reduce individual reproduction, nor do they modulate the effect of short day length. Taken together, we conclude that novel light : temperature relations do not suppress the pest potential of aphids in a changing climate.

ACKNOWLEDGEMENTS

We thank Grit Kunert, MPI Jena, for provision of the aphid clone, and we thank Christie Bahlai and two anonymous reviewers for useful comments on the manuscript.



Larva of the marmalade fly, *Episyrphus balteatus*, one of the organisms used in this study.

CHAPTER IV: DAY LENGTH CONSTRAINTS OF APHID PREDATORS

This chapter has been submitted to Insect Science as:

Joschinski J, Kiess T, and Krauss J. Day length constrains the time budget of aphid predators.

ABSTRACT

Phenology shifts and range expansions cause organisms to experience novel day length – temperature correlations. Depending on the temporal niche, organisms may benefit or suffer from changes in day length, thus affecting phenological adaptation. We assessed the impact of day length changes on larvae of *Chrysoperla carnea* (Stephens) and *Episyrphus balteatus* (De Geer), both of which prey on aphids. Larvae of *E. balteatus* are night-active, whereas those of *C. carnea* appear to be crepuscular. We subjected both species in climate chambers to day lengths of 16:8 LD and, to circumvent diapause responses, 20:4 LD. We recorded development times and predation rates of both species.

E. balteatus grew 13% faster in the 16:8 LD treatment and preyed on significantly more aphids. In contrast, *C. carnea* grew 13% faster in the 20:4 LD treatment and higher predation rates in 20:4 LD were marginally significant. Our results show that day length affects development and predation, but that the direction depends on species. We argue that the differences are linked to differences in diurnal rhythms.

INTRODUCTION

The rotation of earth around itself causes the change of day and night, and most species are adapted to this predictable change by being themselves rhythmic. This specialization to one temporal niche causes evolution of a whole range of traits (e.g. Barton, 1998). For example, when endothermia and nocturnality evolved in mammals, the change in life style was accompanied by large-scale changes in vision, UV tolerance and tactile senses (Gerkema et al., 2013). Although temporal niches can be reversed by evolution (Mrosovsky & Hattar, 2005), individuals rarely switch activity times without fitness costs (Kronfeld-Schor & Dayan, 2003). Therefore, diurnal rhythms can constrain activity patterns.

If animal activity can only occur during day or during night, then day length can constrain the time budget. For example, feral goats on the Isle of Rum are limited by their strictly diurnal rhythm, and cannot expand to more northern latitudes (Dunbar & Shi, 2013). More generally, day length can limit the geographic distribution of species, and contribute to declining species diversity towards the poles (Anderson & Jetz, 2005), though the literature on such day length constraints is biased towards endothermic vertebrates. Day length additionally varies over the course of the year. Seasonal organisms evolved mechanisms to cope with changes in temperatures and day length such as hibernation, but these mechanisms can limit species in their seasonal timing.

Climate change alters the onset and duration of seasons (IPCC, 2014; Thackeray et al., 2016), making the current responses to shorter days non-adaptive. Changes in phenology will thus likely alter diapause and other seasonal strategies. Furthermore, climate change causes range expansion to previously cooler regions (Parmesan & Yohe, 2003), and the magnitude of day length constraints in range expansions is largely unexplored.

When climate change causes range expansions and phenology shifts, different life history stages will be exposed to a certain day length. It is unknown how organisms cope with altered day length – temperature correlations, because overwintering and other seasonal strategies currently conceal the potential constraints of altered day lengths. The aim of this study is thus to study day length effects in a system without evoking adaptive seasonal responses.

We have previously shown that short days affect fitness of the pea aphid, using an aphid line that has secondarily lost its seasonal response (Joschinski et al., 2015). The total loss of seasonal responses might, however, not be realistic in other species groups. Hence, in this study we use the two aphid predators *Episyrphus balteatus* (De Geer) and *Chrysoperla carnea* (Stephens), both of which exhibit diapause as adults (Tauber & Tauber, 1969; Hondelmann & Poehling, 2007). We circumvent diapause by testing long-day conditions (16:8 LD) against even longer days (20:4 LD), a scenario that simulates northward range expansion.

MATERIALS AND METHODS

We tested how day length influences development and predation rates of *Chrysoperla carnea* (Stephens, 1863) and *Episyrphus balteatus* (De Geer, 1776). We obtained both species in advanced egg stage (Katz Biotech, Baruth, Germany, www.katzbiotech.de) and reared them individually from egg to adult. All experiments were conducted in climate chambers (Sanyo MLR-352H) at 18°C and 80% humidity, using day lengths of 16:8 LD and 20:4 LD.

Food source

As food source for *E. balteatus* and half of the *C. carnea* individuals, we used the aphids *Acyrthosiphon pisum* (Harris, 1776), *Metopeurum fuscoviride* (Stroyan, 1950) and *Aphis fabae* (Scopoli, 1763). We used a mixture of aphids instead of a single species, because diet diversity generally maximizes fitness of the predators (Evans et al., 1999). To exclude effects of day length on the aphids, the other half of *C. carnea* received immobile eggs of the moth *Sitotroga cerealella* (Olivier, 1789; Katz Biotech, Baruth, Germany, www.katzbiotech.de).

A. pisum stock cultures were reared in climate chambers (20°C, 80% humidity, 16:8 LD) at high density on two to six week old *Vicia faba* 'Fuego' plants. *M. fuscoviride* and *A. fabae* were collected from *Tanacetum vulgare* and *Cirsium vulgare* with a fine brush on the day of use.

Experiment

We distributed 120 individual *E. balteatus* eggs and 120 individual *C. carnea* eggs randomly to two climate chambers with day lengths of 16:8 LD and 20:4 LD. In both day length treatments we placed 60 individual *E. balteatus* eggs in separate plastic tubes (5.5 x 1 cm), and 60 *individual C. carnea* eggs in separate Petri dishes (3.5 cm diameter, 1 cm high). We supplied all *E. balteatus* eggs and 30 eggs of *C. carnea* with *A. pisum* aphids (5-10 adults and nymphs). The 30 remaining *C. carnea* individuals received 400-500 eggs of *S. cerealella*. Petri dishes were then sealed with parafilm, and plastic tubes were covered with a cotton plug. After hatching, which took 1-3 days for *C. carnea* and 1-2 days for *E. balteatus*, we fed all predators *ad libitum*, by providing daily 5-10 *A. pisum*, 20-25 *M. fuscoviride*, or 20-25 *A. fabae* aphids. The predators that were raised on moth eggs received 400-500 new eggs two to three times per week instead. We recorded developmental stages and mortality daily.

To determine predation rates, we deviated from this feeding scheme twice (*E. balteatus*) or three times (*C. carnea*). *E. balteatus* was fed with 10 *A. pisum* aphids on day 3 and we recorded the number of surviving aphids one day later. The measurement was repeated on day 6 with 20 aphids. Predation rates of aphid-fed *C. carnea* were measured once per molting stage, on days 9, 14 and 19. On these days, we supplied 10, 15 and 20 aphids, respectively.

Statistics

We used R Version 3.1.1 (R Core Team, 2014) for all analysis. We compared mortalities in the two day length treatments with Chi-square tests of goodness of fit, and hatching and development times with Welch's two-sample t-test.

To compare predation rates among treatments, we applied a generalized linear mixed-effects model with Poisson-distributed errors (package lme4, Bates et al., 2015). We included the terms photoperiod and age as fixed factors, an interaction of age and photoperiod, and individual as random term. We included hatching date as additional covariate, so the full model was:

predation ~ photoperiod * age + hatching , random = 1|ID (eq. 1)

Statistical significance was assessed by comparing the full model with reduced models. We avoided likelihood ratio tests that rely on an asymptotic $\chi 2$ test, as these are not recommended for GLMMs with small sample sizes (Halekoh & Højsgaard, 2014). Instead, we created a null distribution in a parametric bootstrap approach (B = 1000), and calculated p as the fraction of samples that exceeded the observed LRT value (package pbkrtest, Halekoh & Højsgaard, 2014). We confirmed the validity of the results with $\chi 2$ – based LRT tests and with bootstrap confidence intervals of the fixed effects. Sample sizes (see results) are summarized in Table IV.1.

Table IV.1. Sample size used in statistical analysis

	Mortality	Development	Predation rate
E.balteatus			
16:8 LD	53	23	23
20:4 LD	52	22	22
C.carnea			
16:8 LD	60	26	12
20:4 LD	60	25	12

RESULTS

A) Episyrphus balteatus

Within the first few hours after hatching, seven larvae were lost in 16:8 LD, and eight in 20:4 LD (Table IV.1). 32 of the remaining 53 eggs hatched successfully in the 16:8 LD treatment, but two larvae died subsequently, whereas 30 out of 52 eggs hatched in 20:4 LD. Hence, 30 out of 53 larvae (57.7%) died in 16:8 LD, and 30 out of 52 (56.6%) died in 20:4 LD. Mortality rates were not significantly different among treatments ($\chi^2 = 0.0035$, $p = 0.95$). Three further larvae died during pupation in 16:8 LD, and five in 20:4 LD, reducing the sample sizes to 20 and 17 respectively.

The larvae hatched in both treatments after 1-2 days, and we found no effect of day length on hatching times ($t = 0.13$, $p = 0.90$). Day length affected both larval ($t = -2.95$, $p < 0.01$) and pupal development time ($t = -2.95$, $p < 0.01$), and in total, development took two days longer in 20:4 LD ($t = -5.85$, $p < 0.001$; Fig. IV.1).

To measure predation rates, we supplied each individual with 10 (first measurement) or 20 (second measurement) aphids. One third of the individuals depleted all aphids in the second measurement (10 in 16:8 LD, 5 in 20:4 LD), so we may underestimate predation rates, particularly in 16:8 LD. Aphid predation (table IV.2, Fig. IV.2) did not depend on hatching date ($p = 0.62$), but increased significantly with age ($p < 0.01$). Predation was significantly higher in the 16:8 LD treatment ($p < 0.01$), despite being likely underestimated. The interaction of photoperiod and age was not significant ($p = 0.77$). These results conform well to simple LRT test results (photoperiod: $p < 0.01$, interaction: $p = 0.77$).

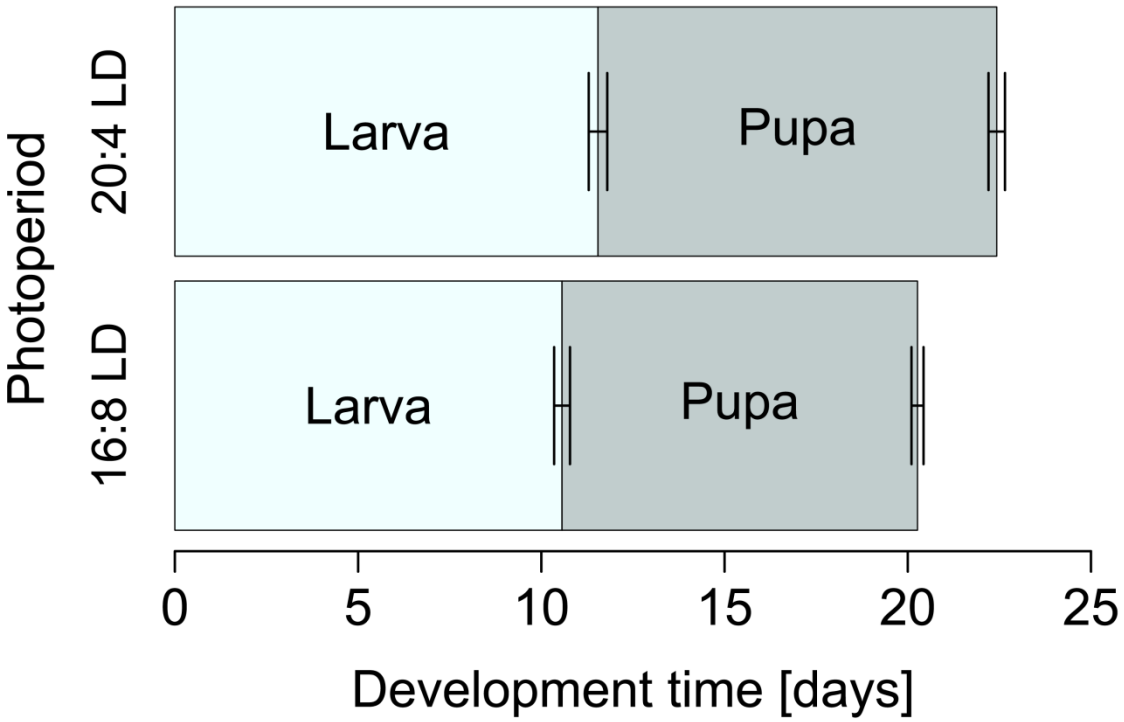


Fig. IV.1: Mean development times of *E. balteatus* in two day length treatments. Error bars indicate S.E.M.

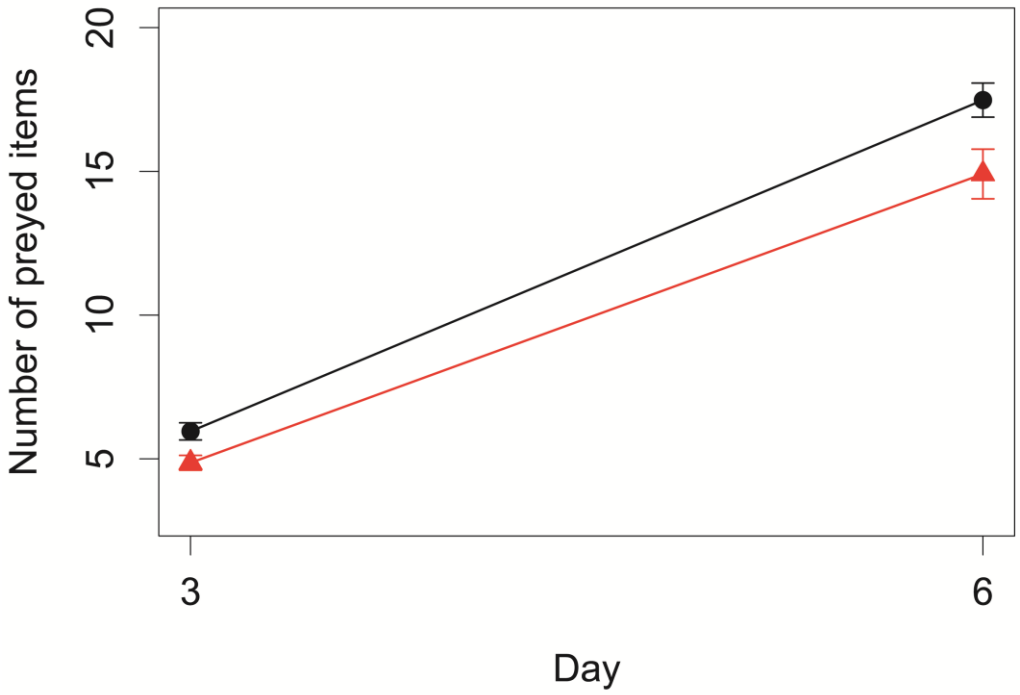


Fig. IV.2: Predation rates of *E. balteatus* under two day length treatments, fed with 10 *A. pisum* aphids on day 3, and 20 aphids on day 6. Circles: 16:8 LD, triangles: 20:4 LD.

Table IV.2. Predation rates of *Episyrphus balteatus* and *Chrysoperla carnea*. The original model (predation ~ photoperiod * age + hatching, random =1| ID) was reduced with the help of a parametric bootstrap test. The bootstrap approach does not rely on a χ^2-distribution, so no χ^2-values or degrees of freedom are given. Asterisks indicate significance level:
$p < 0.01$ ******; $p < 0.05$ *****; $p < 0.10$ **(*)**.

Episyrphus balteatus	p	
photoperiod : age	0.75	
hatching	0.59	
age	<0.01	**
photoperiod	<0.01	**

Chrysoperla carnea	p	
photoperiod : age	0.92	
hatching	0.76	
age	<0.01	**
photoperiod	0.078	(*)

B) Chrysoperla carnea

32 out of 60 eggs hatched in each chamber, 15 of which were fed with aphids, and 17 with *Sitotroga* eggs. Six larvae (3 aphid-fed, 3 *Sitotroga*-fed) died in the 16:8 LD treatment, and seven (3 aphid-fed, 4 *Sitotroga*-fed) in 20:4 LD. No larvae escaped in either treatment, thus mortality rates were 56.7% (34 out of 60) in 16:8 LD, and 58.3% (35 out of 60) in 20:4 LD ($\chi 2 = 0.0092$, $p = 0.92$).

Hatching times differed between the two treatments ($t = 7.35$, $p < 0.001$), and larvae hatched 1.1 ± 0.31 day earlier in 20:4 LD. Hatching times played, however, no role in later analysis (Table IV.2). When fed with *Sitotroga* eggs (Fig.IV.3A), day length affected all larval stages ($t = 2.98$, $p < 0.01$; $t = 2.74$, $p < 0.05$; $t = 3.23$, $p < 0.01$) as well as pupal development time ($t = 7.76$, $p < 0.001$), and *C. carnea* developed in total 6.25 days earlier in 20:4 LD ($t = 9.41$, $p < 0.001$). The larvae that were fed with aphids (Fig. IV.3B) responded to day length in their first larval stage ($t = 5.14$, $p < 0.001$; $t = 1.18$, $p = 0.25$; $t = 1.67$, $p = 0.11$) and pupation period ($t = 5.01$, $p < 0.001$), causing a decrease in development times of 5.5 days ($t = 5.59$, $p < 0.001$).

Aphid predation (Table IV.2, Fig. IV.4) increased significantly with age ($p < 0.01$). Photoperiod had a marginally significant effect ($p = 0.076$), causing higher predation rates in the 20:4 LD treatment, whereas the interaction of photoperiod and age was not significant ($p = 0.90$). These results were very similar to a standard LRT test (photoperiod: $p = 0.08$; interaction: $p = 0.92$). In contrast to *E. balteatus*, aphids were provided in excess, and in no case depleted.

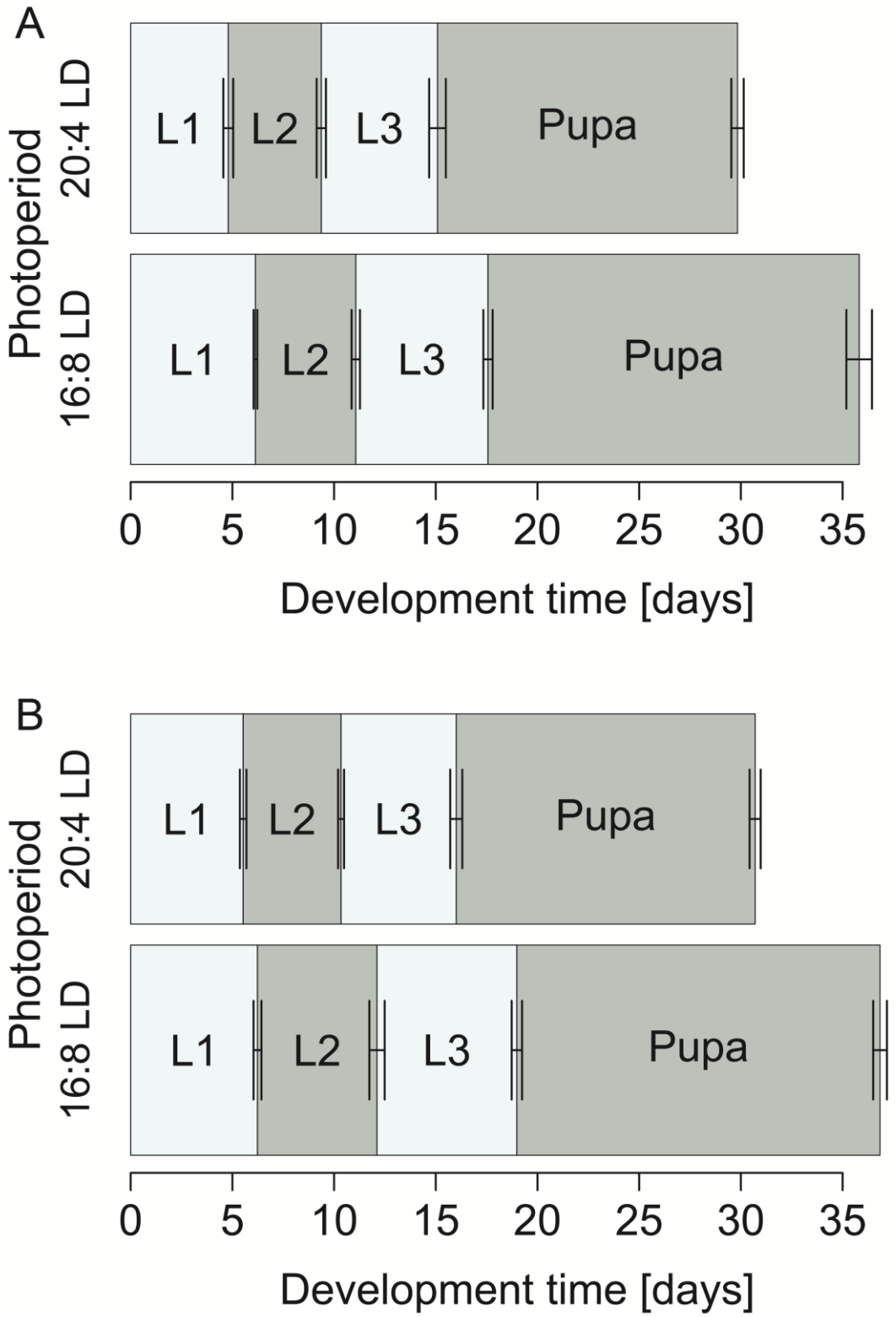


Fig. IV.3: Mean development times of *C. carnea* in two day length treatments. Panel A) *C. carnea* was fed *ad libitum* with an aphid mix. Panel B) *C. carnea* was fed *ad libitum* with eggs of the moth *Sitotroga cereallela*. Error bars indicate S.E.M..

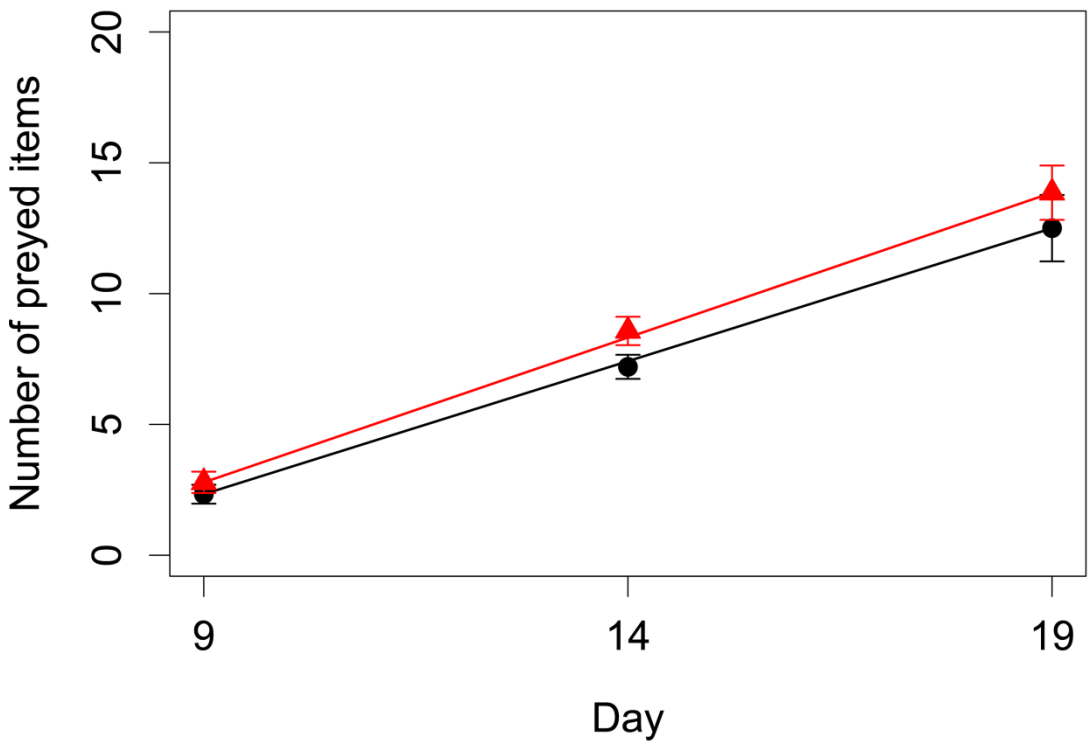


Fig. IV.4: Predation rates of *C. carnea* under two day length treatments, fed with 10,15 and 20 aphids on days 9, 14 and 19. Circles: 16:8 LD, triangles: 20:4 LD.

DISCUSSION

Our study on fitness constraints of altered day length showed that *E. balteatus* benefits from longer nights, whereas *C. carnea* benefits from longer days.

Photoperiod did not affect hatching in *E. balteatus*, but in *C. carnea*. This is surprising given the advanced stage of the eggs at the start of the experiment. Long days promoted hatching, and we hypothesize that day length acts as cue for emergence. This finding is in line with a comparable study of day length effects on *C. carnea*, which found earlier hatching under long days (Qadeer et al., 2012). In principle, these differences in age could have influenced our estimates of development times or predation rates. We find it, however, unlikely that age differences played a major role, because our full model of aphid predation included age as covariate, but age was then removed as non-significant. Hence, the differences in hatching times of less than 24 hours were potentially too small to have an effect.

Development was more notably influenced by day length in both species. While *C. carnea* developed 13% faster under longer days, *E. balteatus* developed 13% faster under long nights. Predation rates were affected in the same way, as predation increased with day length in *C. carnea*, but decreased in *E. balteatus*. Overall, we have shown that changes in day length affect development and predation of two aphid predators, but that the direction depends on species. This study is to our knowledge the first to evaluate day length effects in aphid predators while circumventing seasonal responses.

The observed day length constraints may affect how species cope with climate change. Many species already responded to climate change with range expansions (Parmesan & Yohe, 2003) and phenology shifts (Thackeray et al., 2016), but changes in geographic distribution or phenology affect the available day length. Thus, day length constraints can slow down adaptation to climate change. Especially the observed effects on development time have direct implications for fitness, as the onset of reproduction is the most important predictor of population growth (Cole, 1954). Phenological adaptation is already limited by the rate of microevolution in some species (Gienapp et al., 2013), so any further constraints that hamper the evolution of phenology shifts could reduce the ability to track climate change. Therefore, the effects of day length, whether positive or negative, need to be considered when assessing responses to climate change.

We assume that the cause of day length constraints is the limited time budget due to activity patterns. Indeed, *E. balteatus* is night-active (Holmes, 1984; Ankersmit et al., 1986), which explains the benefit the species derives from longer nights. In contrast to *E. balteatus*, *C. carnea* does not appear to have a clear diurnal pattern. Three independent studies, all constrained by

limited sample size, indicate a bimodal distribution of activity (Schotzko & O'Keeffe, 1989; Joachim & Weisser, 2013; Woltz & Landis, 2014), though the timing of the peak activities varies among the three studies. Thus, the constraints of shorter days are not explained by strictly diurnal activity. Further studies on the diurnal rhythm of *C. carnea* are needed to determine the effect light has on predation by this important biocontrol agent.

We conclude that the two aphid predators react in opposing directions to day length changes. This finding demonstrates that a realistic assessment of day length effects needs to consider the whole trophic network. We showed in earlier studies that aphids have an independent diurnal rhythm (Joschinski et al., 2016), and that aphids suffer from shorter day length (Joschinski et al., 2015). Because aphids possess various ways to defend against predation, e.g. by emission of alarm pheromone (Kislow & Edwards, 1972) and by escape behaviors (Losey & Denno, 1998), one might have expected that aphids are easier to subdue when less active, i.e., under shorter days. However, the interaction term of day length and age was for both predators in our study not significant, indicating that the predator efficiency was similar and that the food quality did not differ among the day length treatments. By confining aphids and predators to a small space, we potentially rendered all defense and escape behaviors ineffective in the experiment. In line with this finding, the results of the predators interacting with living aphids do not differ qualitatively from those reared on immobile eggs. Future studies are thus needed to test how day length affects species interactions under natural conditions.

CONCLUSIONS

We have shown that shorter days, which are associated with phenology changes, are beneficial for one aphid predator, but detrimental to another. This finding highlights the need to consider the whole trophic network when assessing fitness effects of phenology shifts.

ACKNOWLEDGEMENTS

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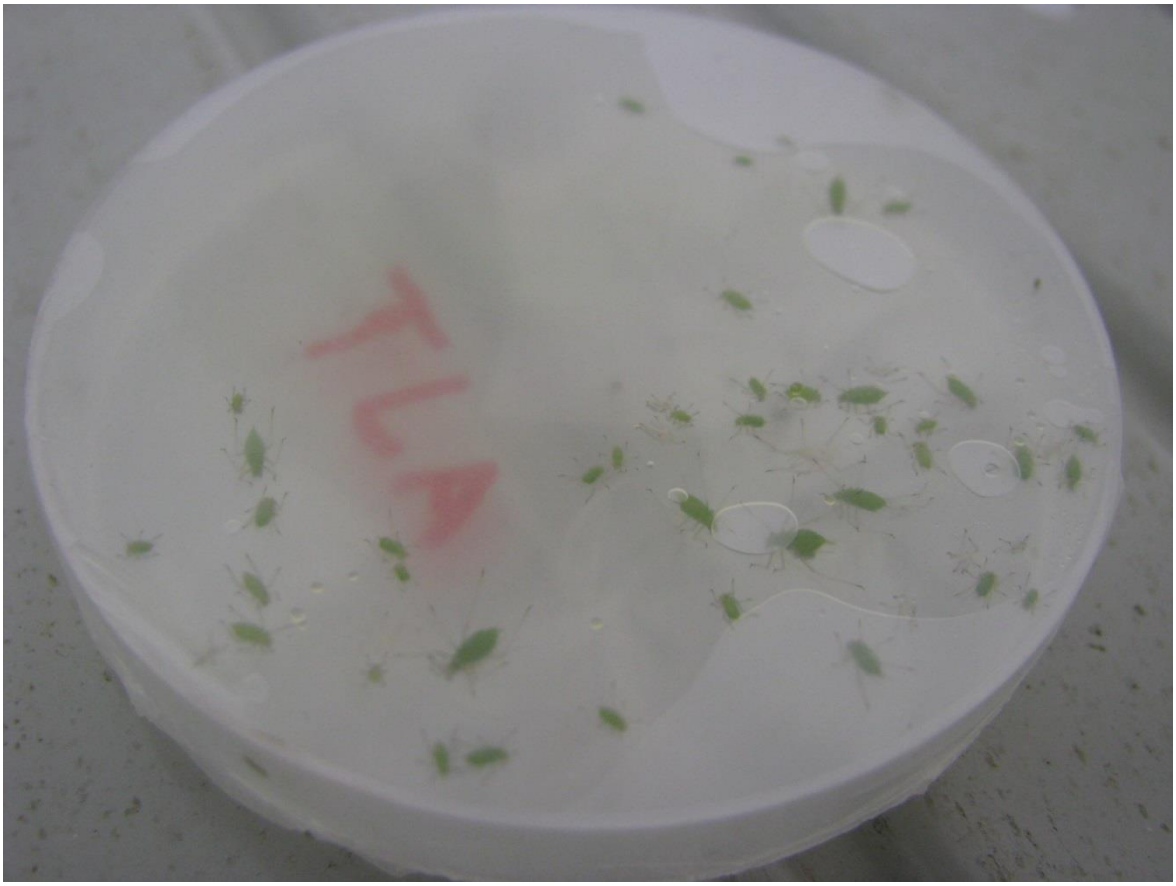

A colony of *Acyrthosiphon pisum*, reared on a holidic artificial diet that consists of sugar, amino acids and trace metals.

CHAPTER V: DIURNAL RHYTHM OF APHIDS

This chapter was published as:

Joschinski J, Beer K, Helfrich-Förster C and Krauss J. 2016. Pea aphids (Hemiptera: Aphididae) have diurnal rhythms when raised independently of a host plant. *Journal of Insect Science* **16.**

ABSTRACT

Seasonal timing is assumed to involve the circadian clock, an endogenous mechanism to track time and measure day length. Some debate persists however, and aphids were among the first organisms for which circadian clock involvement was questioned. Inferences about links to phenology are problematic, as the clock itself is little investigated in aphids. For instance, it is unknown whether aphids possess diurnal rhythms at all. Possibly, the close interaction with host plants prevents independent measurements of rhythmicity.

We reared the pea aphid *Acyrthosiphon pisum* (Harris) on an artificial diet, and recorded survival, moulting and honeydew excretion. Despite their plant-dependent life style, aphids were independently rhythmic under light-dark (LD) conditions. This first demonstration of diurnal aphid rhythms shows that aphids do not simply track the host plant's rhythmicity.

INTRODUCTION

Throughout latitudes and altitudes day- and night-time temperatures and their respective durations vary, and species are adapted to make best use of the temporal niches via diurnal or nocturnal activity (Bennie et al., 2014). Diurnal rhythms can dictate whether species meet, and can contribute to presence or absence of biotic interactions (Stich & Lampert, 1981; Fleury et al., 2000). Due to the relevance of a correct timing, nearly all organisms examined so far possess an endogenous mechanism called circadian clock (Moore-Ede et al., 1982; but see Lu et al., 2010), and the circadian clock affects all major physiological processes (Moore-Ede et al., 1982).

One remaining question is whether the clock is involved in seasonal timing (phenology) via day length (photoperiod) measurements (Bünning, 1936). Supporters of clock involvement have proposed two models, which describe how phase relations of clock and environment (external coincidence, Pittendrigh & Minis, 1964), or of multiple clocks (internal coincidence, Pittendrigh & Minis, 1972) could govern photoperiodism. Numerous studies indeed correlated clock gene expression with photoperiodism (Schultz & Kay, 2003), including in hemipterans (Ikeno et al., 2010), but this correlation can be at least partially attributed to research bias in favour of clock genes (Bradshaw & Holzapfel, 2010). Hence, despite accumulating correlative evidence the debate is still not fully settled (Danks, 2005).

An alternative to clock involvement in photoperiodism is the hour glass model (Garner & Allard, 1920). In this model, steady, clock-independent accumulation of a molecule triggers a response upon reaching a threshold. Aphids played a prominent role in the discussion of clock involvement in photoperiodism, as they were seen as first evidence for such an hour glass model (Lees, 1973). Careful re-evaluation contradicted this view, and suggested that aphid photoperiodism depends on the circadian clock (Hardie & Vaz Nunes, 2001). The clock was proposed to damp quickly, i.e. disappear within few cycles. However, very little empirical data is available about damping or other properties of the aphid clock itself, let alone studies on how the clock affects aphid behaviour. Before settling the argument on circadian clock involvement in photoperiodism, the first logical question is whether aphids have a diurnal rhythm driven by an endogenous clock.

The lack of research on diurnal rhythms of aphids may in part be explained by the high degree of food specialization, which complicates studies of an independent rhythm. Aphids are known for their remarkable phenotypic plasticity, and asexual forms (morphs) can bear sexual offspring if induced by a short photoperiod (Lees, 1973). Because the sexual offspring is less dependent on host plants, experiments with various aphid species have been conducted on such sexual morphs (Eisenbach & Mittler, 1980; Thieme & Dixon, 1996). However, if the ultimate aim is the link of circadian clock and photoperiodism, tests on long-day (asexual) aphids are needed. While some

experiments have been also conducted on asexual morphs, the aphids were always held on living plants (Gomez et al., 2006; Cortes et al., 2010; Taylor et al., 2012), so the changing C:N ratio of the plant might have entrained the aphid, i.e. food has reset the circadian clock. Only one study has elegantly disentangled plant and aphid clocks by subjecting both to different light-dark rhythms (Hodgson & Lane, 1981), but such a protocol cannot be extended to constant darkness. Instead of relying on different morphs or on plants we chose to raise aphids independent of their host plants. Even though artificial diets have been developed for the pea aphid (Febvay et al., 1988), they have never been used to resolve this problem. We thus asked whether aphids have a diurnal rhythm by measuring honeydew excretion and moult on an artificial diet, as these phenomena likely represent clock output.

METHODS

Aphids are relatively immobile, so monitoring locomotor activity is currently not possible. We therefore decided to quantify feeding and moulting. To do so, we counted exuviae and honeydew drops, which are the excess sugar excreted after feeding from the plant sap. Although artificial diets are well-suited to monitor activity uncoupled of the host, there is also a considerable limitation: Artificial diets deteriorate within two to three days (van Emden & Harrington, 2007), so the diet has to be renewed within less than two days. Furthermore, even with regularly renewed diet, food intake is reduced on artificial diets (van Emden & Harrington, 2007), and we noticed a relatively low amount of honeydew production of about 1 drop per aphid and day, requiring large sampling intervals.

These problems prompted us to design an admittedly complex experiment (see also Fig. V.1). Aphids were held continuously under 16:8 LD (light:dark) to avoid induction of sexual morphs. We renewed the diet every 1.5 days, either shortly after lights-on, or shortly before lights-off, and counted accumulated honeydew and exuviae. Thus, the aphids experienced either one night (16 h light + 8 h darkness + 12 h light = 28:8 LD, treatment ‘L’) or two nights (4L + 8D + 16L + 8D = 20:16 LD, treatment ‘D’) between measurements. This feeding protocol also avoided entrainment (synchronisation of the clock) with the feeding rhythm, as food was provided in the morning or evening in an alternating manner. To reduce the influence of any diurnal disturbance, we replicated the experiment in another chamber, which was phase-shifted by 12 hours. We hence expected subsequent increases and decreases in activity with opposing patterns in the two chambers (see also Fig. V.1).

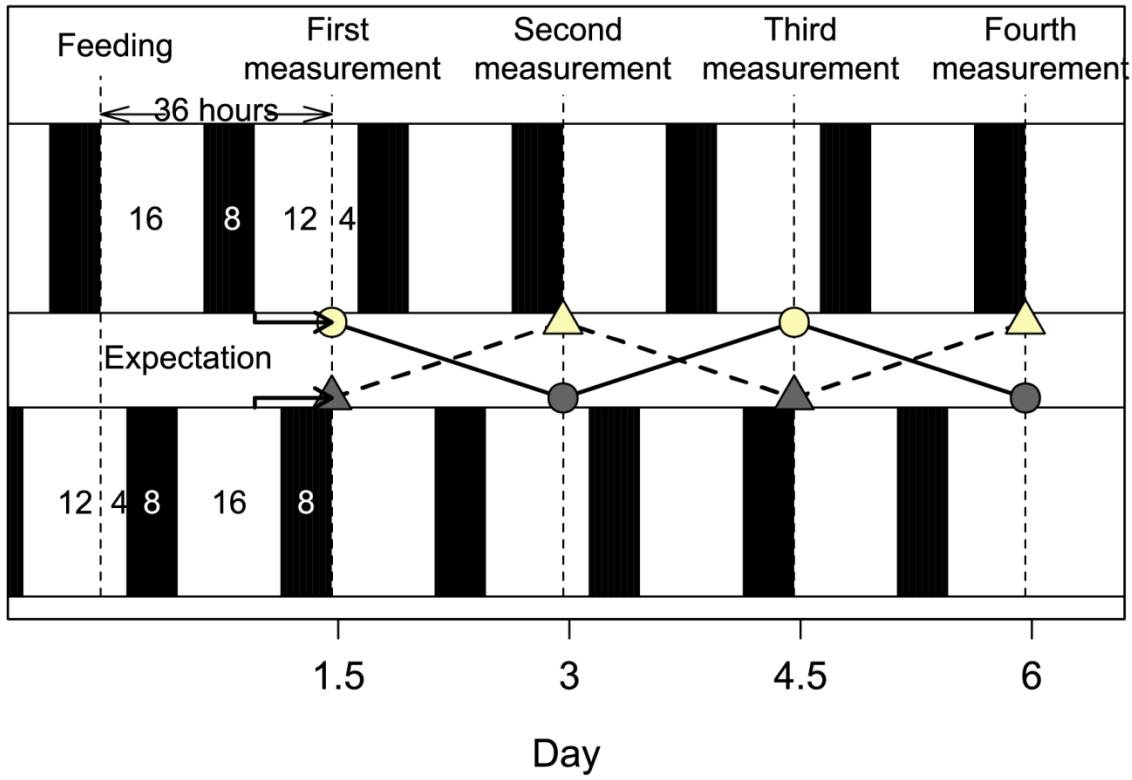

Fig. V.1: Experimental setup. The experiments were conducted in parallel in 2 climate chambers. Both chambers were set to continuous 16:8 h light-dark rhythms but differ in phase. Over the first 36 hours after start of the experiment (first dashed line, "feeding") one chamber received 28 hours of light (16L + 8D + 12L, treatment 'L'), whereas the other received 20 hours of light (4L + 8D + 16L + 8D, treatment 'D'). Accumulated honeydew and exuviae were counted at the end of the 36 hour period (dashed line). Measurements were repeated four times, so that the aphids in each chamber received alternating amounts of night time (8-16-8-16 in the upper chamber). Accumulated honeydew and exuviae (activity) of the two chambers (circles and triangles) were expected to depend on treatment (bright vs. dark), and hence to be in opposing directions in the two chambers.

The ratio of the treatments L/D depends on the amount of honeydew produced during lights-on (x) and during lights-off (y):

$$\frac{L}{D} = \frac{28x + 8y}{20x + 16y} \qquad \text{(eq. 1)}$$

Therefore we expected the ratio of the two treatments to range between 0.5 (x=0, night active) and 1.4 (y=0, day active), and to be 1 if aphids prefer neither day nor night (x=y). We repeated the measurements four times. We provided a holidic diet with 20% w/v sucrose and defined amounts of amino acids, vitamins and trace metals. The diet is based on diet A0 by Febvay et al. (1988), but uses 10 mM nicotinic acid instead of nicotine amide; a full recipe can be found in Supplementary Table V.S1. We fed it to clone LL01, an asexual green alfalfa biotype originally from the Lusignan area, kindly provided by G. Febvay (INRA Lyon, France). Following a technique by Mittler & Dadd (1963), aphids were held in 35x10 mm Petri dishes, which were covered with two stretched parafilm M membranes with 250 µl diet in between. Per chamber we placed 14 replicates of 30 newly born nymphs in Petri dishes. The parents of the nymphs were light-entrained under 16:8 LD (i.e. the circadian clock was synchronised with the light-dark cycle), but reared on plants. We started the first measurement period at four days age. Every 1.5 days (during lights-on in both chambers) we placed surviving nymphs into new Petri dishes with new food and counted the accumulated honeydew and exuviae. We counted the number of honeydew drops but did not estimate the volumes due to the low visibility and the small sizes; we noticed however no trend in drop diameters, and variability in drop volumes can be considered low (Auclair, 1958). The two climate chambers (Sanyo MLR-H series) provided 18.1 ± 0.9 °C and 81.3 ± 2.8 humidity and a 16:8 LD rhythm at 19.7 ± 0.7 klux.

Statistics were performed with R 3.1.1 (R Core Team, 2014). We applied a mixed-effects model including chamber, time and their interaction as factors, and Petri dish as random term. As we expected alternating slopes (Fig. V.1), a significant interaction term with reversing slopes would evidence rhythmicity. We corrected honeydew excretion for the number of surviving aphids. We additionally corrected honeydew excretion for moulting aphids, which are not expected to produce honeydew. This yielded the combined activity estimate Drops/(Survivors-Exuviae).

RESULTS

Honeydew excretion was overall low with 1-3 drops/aphid per 1.5 days, but the nutrient uptake was sufficient for experimental animals to develop into adults and to survive for two weeks. Survival declined over time (65%-89% survival rates between measurements), leaving on average 38% (11.4 ±0.4 aphids) at the fourth measurement (see Supp. Figure V.S1). Under 16:8 LD conditions, survival, moulting and honeydew excretion individually did not significantly alternate with changing treatments, but the slopes of the combined activity estimate crossed significantly (i.e. significant statistical interaction, Table V.1; Table V.2; Fig. V.2). This interaction conforms to our prediction of diurnal rhythmicity. In the L treatments (with 28 h light in 36 h), the median of observed activity (drops per non-moulting survivor) was 1.80 drops, 31% higher than in the D treatments (with 20 h light in 36h, 1.37 drops). The ratio of L/D = 1.31 allows estimates on how active aphids were during lights-on and during lights-off (x and y in eq. 1). For instance, if aphids were purely day-active (x = 1, y = 0), one would expect activity for 28 hours in the L treatment and for 20 hours in the D treatment, so that the ratio L/D between the treatments would be 28/20 = 1.4, or 40% higher in the L treatment. Solving eq. (1) with L/D = 1.31 yields 1 y = 7.2 x. We conclude that aphids are seven times more active during day than during night.

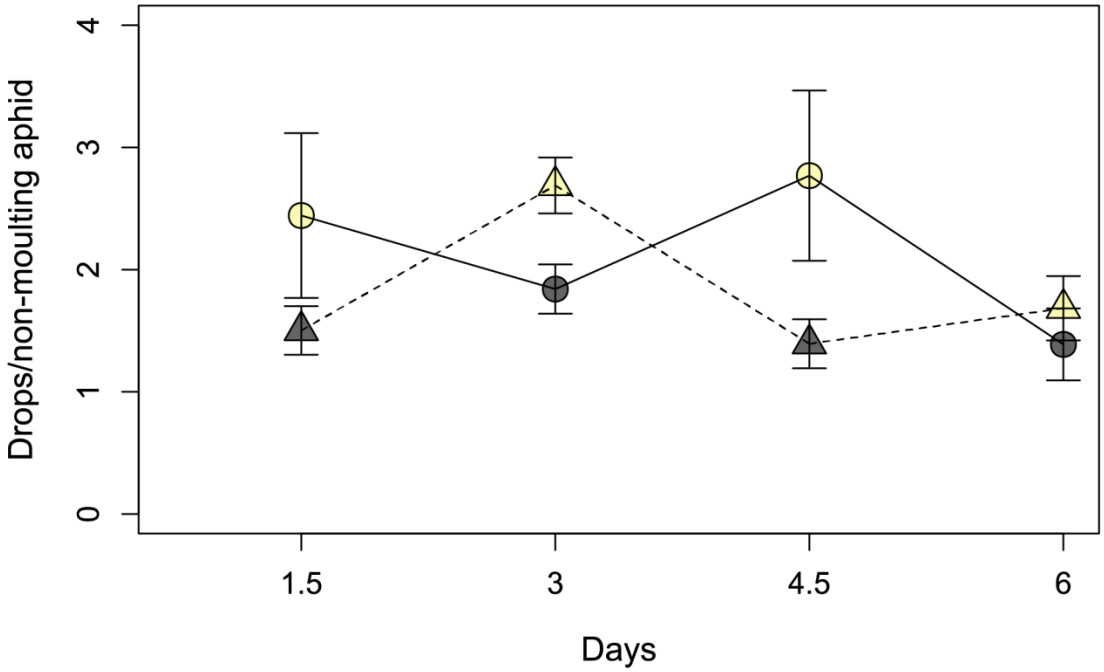

Fig. V.2: Honeydew excretion per non-moulting aphid in changing day:night ratios (see also Fig. V.1). Colour coding is the same as in Fig. 1, i.e. aphids from the two chambers (circles and triangles) that received the L treatment (28 h light, 8 hours darkness) are presented in yellow, whereas aphids in the D treatment (20 h light, 16 hours darkness) are presented in grey. Error bars indicate s.e.m.

Table V.1: ANOVA results. The three responses aphid survival, moulting and honeydew excretion were combined to one estimate that expresses activity as honeydew excretion per non-moulting survivor. We expected a significant interaction of chamber and time (see main text).

Response	Factor	df	F	p	Significance level[a]
survival	chamber	1,26	1.40	0.25	
	time	3,77	85.70	<0.0001	***
	chamber : time	3,77	1.59	0.20	
moulting	chamber	1,26	1.19	0.29	
	time	3,77	3.24	0.03	*
	chamber : time	3,77	1.77	0.16	
honeydew	chamber	1,26	0.08	0.78	
	time	3,69	5.37	<0.01	**
	chamber : time	3,69	2.43	0.07	‘
activity	chamber	1,26	0.99	0.33	
	time	1,69	1.27	0.29	
	chamber : time	1,69	3.02	0.04	*

[a]Significance levels: *** p<0.001; ** p<0.01; * p<0.05; ‘ p<0.1

Table V.2: Means (± s.e.m.) of estimates for diurnal rhythms. Activity is the combined estimate of honeydew excretion per non-moulting survivor. The four measurements were taken after 1.5, 3, 4.5 and 6 days, and correspond to the dashed lines in Fig. V.1. Treatment L corresponds to 28h light and 8 h darkness, Treatment D corresponds to 20 h light and 16 h darkness as described in Fig. V.1 and in the main text.

Response	Treatment	First measurement	Second measurement	Third measurement	Fourth measurement
survival	L	25.79 (± 1.45)	21.57 (± 1.56)	12.93 (± 0.90)	12.64 (± 0.95)
	D	26.85 (± 1.51)	19.86 (± 1.06)	18.07 (± 1.26)	10.14 (± 0.93)
moulting	L	11.93 (± 1.08)	6.57 (± 0.79)	6.00 (± 0.59)	5.79 (± 0.68)
	D	10.31 (± 1.21)	5.79 (± 0.52)	6.57 (± 0.64)	3.64 (± 0.37)
honeydew	L	28.46 (± 3.91)	37.86 (± 2.95)	16.07 (± 2.95)	12.00 (± 1.57)
	D	24.46 (± 3.02)	23.09 (± 1.73)	14.46 (± 1.51)	8.00 (± 1.23)
activity	L	2.44 (± 0.50)	2.69 (± 0.17)	2.77 (± 0.52)	1.68 (± 0.20)
	D	1.50 (± 0.14)	1.84 (± 0.15)	1.39 (± 0.15)	1.39 (± 0.22)

DISCUSSION

The experiment is to our knowledge the first to measure aphid diurnal rhythms independent of host plants by feeding artificial diets. Although independent diurnal rhythms have been observed in sexual morphs, after photoperiodic induction (Eisenbach & Mittler, 1980; Thieme & Dixon, 1996), in all studies on asexual morphs aphids were reared on living plants (Gomez et al., 2006; Cortes et al., 2010; Taylor et al., 2012). Aphids can be described as plant parasites (The International Aphid Genomics Consortium, 2010), so aphids might well hitch-hike the plant rhythm instead of using the light-dark cycle (LD). The present study indicates that this is not the case (evidenced by the statistical significant interaction term), and that aphids have diurnal rhythms even on constant food sources.

Common to all studies in various species is an activity peak during day time, and our results show that this is generally also true for the pea aphid. However, further experiments are needed to determine how the activity distributes over the course of the day. Knowledge of the activity pattern of aphids has implications for pest control, because it assists more specific treatment with insecticide in circadian manner (Hooven et al., 2009). Furthermore, it is interesting to know whether the plant modulates the aphid rhythmicity, because exploitation of diurnal changes in host receptivity and quality can lead to coevolution among the circadian clocks (Goodspeed et al., 2012; Martinez-Bakker & Helm, 2015). Changes in diurnal timing are also accompanied by changes in the abiotic conditions, which aphid experience. For example, day – activity might be an explanation of fitness constraints under short days (Joschinski et al., 2015). Overall, our study lays the foundation for future studies on aphid diurnal rhythms and the interaction with their host plants.

We are well aware that the rhythm in diurnal behaviour is no evidence for circadian clock involvement yet, as it needs also continuation (‘free-runs’) under constant conditions (Moore-Ede et al., 1982). Future experiments need to test aphid rhythmicity under constant darkness, and in particular need to quantify how the oscillation of the clock damps out. On the one hand, studies suggest an hour glass mechanism, i.e. no clock involvement, in aphids (Lees, 1973); on the other hand, correlative evidence from other species suggests that this clock mechanism is not the norm (Ikeno et al., 2010). These apparent differences could be united by a quickly damping clock (Hardie & Vaz Nunes, 2001), and a damped clock might be exemplified by damping activity under constant darkness. The molecular mechanism of the aphid clock has been investigated, and some parts of the core clockwork (CRY and the PER/TIM feedback loop) are indeed undergoing accelerated changes (Cortes et al., 2010). Our current protocol, which requires feeding every 36 hours to maintain high honeydew excretion rates, does not yet allow working under constant darkness, because feeding in darkness proved impossible. Hopefully further advances in rearing

methods will allow studying aphid clock properties in depth. Yet, the demonstration of independent diurnal behaviour is a crucial first step in understanding aphid clocks.

CONCLUSIONS

We showed that pea aphids produce honeydew and moult during daytime, and maintain the rhythm independently of host plants. We think that pea aphids are worth investigating for the involvement of clock and photoperiodism in aphid physiology, and our study with artificial diets are a first step in understanding its mechanisms.

ACKNOWLEDGEMENTS

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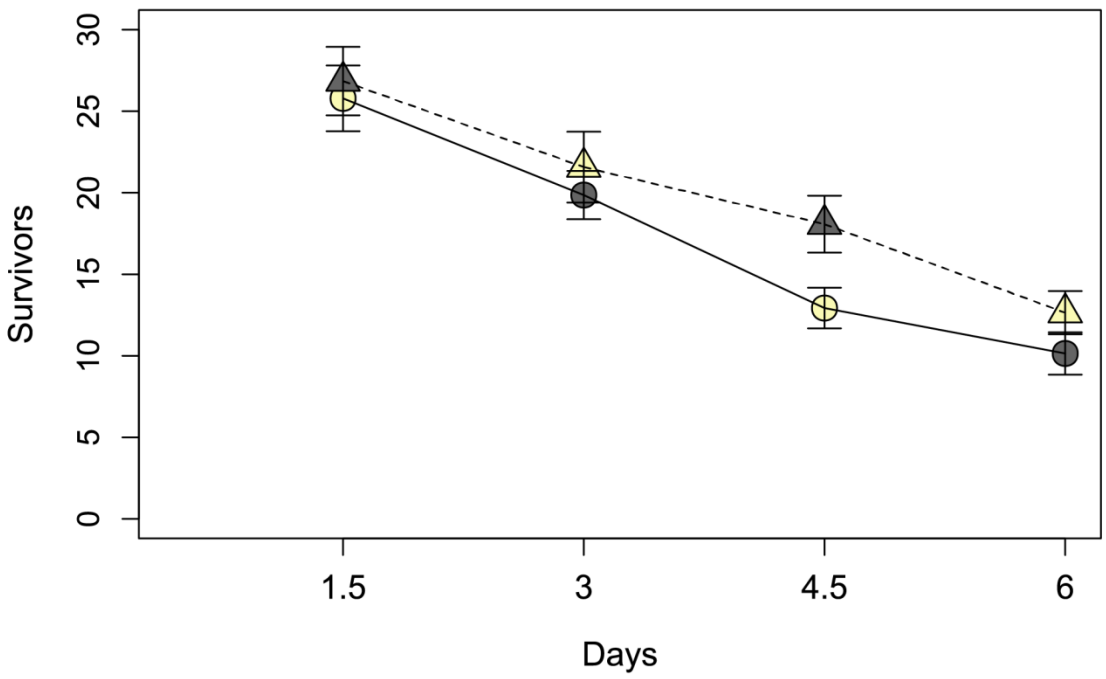
SUPPLEMENTARY MATERIAL



Supp. Figure S1: Aphid survival. The experiment was started with 30 aphids per petri dish, and the mean number of surviving aphids declined during the experiment. Circles and triangles represent the two chambers, and yellow and grey colors represent treatments L and D as in Fig. V.1 and Fig. V.2. Error bars indicate s.e.m.

Supp. Table S1: Recipe for the aphid diet. Further details on the procedure can be found in (Febvay et al. 1988) and (van Emden and Harrington 2007).

Protocol for 500 ml artificial diet

The protocol is based on Febvay et al. (1988), but uses 20% sucrose, and nicotinic acid instead of nicotinamide. Further information about diet preparation and its use can be found in (van Emden and Harrington 2007), or in (van Emden 2009).

The diet has been optimized for the clone LL01, a green alfalfa biotype from the Lusignan area. It might not work for other aphid lines. Even for LL01, rearing beyond a first generation is problematic. 500 ml are sufficient to feed aphids in 1000 petri dishes for 3-4 days.

a) place 350 ml Milli-Q purified water in 1l beaker

b) add 100 g sucrose (± 0.5 g)

c) add amino acids in this order (some ingredients require stirring for up to 30 minutes):

Alanine	893.53	±1	mg
ß-Alanine	31.08	±0.3	mg
Arginine	1224.52	±1	mg
Asparagine H_20	1492.74	±1	mg
Aspartic acid	441.26	±1	mg
Cysteine	185.96	±1	mg
Glutamic acid	746.80	±1	mg
Glutamine	2228.04	±1	mg
Glycine	832.79	±1	mg
Histidine HCl H_20	680.11	±1	mg
Isoleucine	823.73	±1	mg
Leucine	1157.79	±1	mg
Lysine HCl	1755.43	±1	mg
Methionine	361.76	±1	mg
Ornithine HCl	47.06	±0.5	mg
Phenylalanine	1472.67	±1	mg
Proline	646.63	±1	mg
Serine	621.40	±1	mg
Threonine (allo free)	635.80	±1	mg
Tryptophane	213.74	±1	mg
Tyrosine	193.15	±1	mg
Valine	954.27	±1	mg

d) add vitamins, in this order

Calcium citrate	50.00	±0.5	mg
Cholesteryl benzoate [1]	12.50	±0.1	mg
$MgSO_4$	1210.00	±1	mg
p-aminobenzoic acid	50	±0.5	mg
Ascorbic acid	500	±1	mg
Biotin	0.5	±0 [2]	mg
Calcium - panthothenate	25	±0 [2]	mg
Choline chloride	250	±1	mg
Folic acid	5	±0 [2]	mg
i-Inositol	210	±1	mg
Nicotinic acid	50	±0.5	mg
Pyridoxin	12.5	±0 [2]	mg
Riboflavin	2.5	±0 [2]	mg
Thiamine di-HCl	12.5	±0 [2]	mg

[1] Cholesteryl benzoate will not dissolve fully. Stir for 30 minutes.

[2] a small amount may be weighed and dissolved in 1 ml diet, and the appropriate amount in µl calculated and pipetted back into the diet

e) add metals:

$CuSO_4$, 5 H_2O	2.35	±0 [2]	mg
$FeCl_3$, 6 H_2O	22.25	±0.25	mg
$MnCl_2$, 4 H_2O	3.25	±0 [2]	mg
NaCl	12.7	±0 [2]	mg
$ZnCl_2$	4.15	±0 [2]	mg

f) add 1250 ±1 mg KH_2PO_4

g) fill up to 480 ml

h) adjust pH to 7.500 with KOH

i) fill to 500 ml, freeze

Some of the colourants tested in this study.

CHAPTER VI: IMPROVEMENTS TO ARTIFICIAL APHID DIET

This chapter has been submitted to Entomologia Experimentalis et Applicata as:
Joschinski J and Krauss J. Food colouring as new possibility to study diet ingestion and honeydew excretion by aphids.

SUMMARY

Aphids such as *Acyrthosiphon pisum* (Harris) and *Myzus persicae* (Sulzer) (Hemiptera: Aphididae) cause agricultural and economic losses. Thus aphid – host plant interactions are intensively researched. The feeding process is however hard to observe, because aphids do not move while inserting their stylets. Hence, we made ingestion, and honeydew excretion as a proxy for feeding, more visible. We tested 32 colourants in artificial diets on *Acyrthosiphon pisum*, and improved the diet by reducing eight ingredients. One colour, Brilliant Blue FCF, stained both gut and honeydew at a concentration of 0.1 mg/ml, and aphids survived even with high doses for more than one week. Because the food colouring marks aphids that feed on artificial diets and simultaneously colours their honeydew, it may find applications in the study of diverse topics such as virus transmission, insecticide uptake and plant resistance. Moreover, this method can also be applied to *Myzus persicae* and other pest species.

INTRODUCTION

Aphid feeding causes major economic losses (van Emden & Harrington, 2007), as about 10% of the 4700 species are pests (Blackman & Eastop, 2000). Aphids drain resources, transmit plant viruses that can exceed the direct damage (Hogenhout et al., 2008), and coat leaves with honeydew, which reduces photosynthesis (Wood et al., 1988). Thus, much research effort has been directed into aphid feeding and excretion.

The whole feeding process, from initial plant contact to excretion of excess sugar, is economically and ecologically relevant. Aphids first place the mouthparts on the plant surface, then penetrate the plant epidermis with their stylets and probe cells (Powell et al., 2006). Probing aphids can acquire and transmit plant viruses (Pirone & Harris, 1977), but probing can also elicit plant responses (Jaouannet et al., 2014) and expose the otherwise stealthy nature of aphid feeding to the plant defence system (Züst & Agrawal, 2016). If the host is found suitable, aphids start sucking phloem, a sugar-rich but amino acid - deprived diet. Aphids evolved elaborate physiological adaptations to cope with this diet, for example mechanisms for osmoregulation (Jing et al., 2016) and endosymbionts to provide essential amino acids (Douglas, 2015), but endosymbionts may also interfere with plant resistance (Chaudhary et al., 2014). Lastly, the excess sugar of the diet is excreted as honeydew. Honeydew is a valuable energy source for ants, and the ant-aphid mutualism is a keystone interaction in many ecosystems (Styrsky & Eubanks, 2007). Overall aphid feeding and excretion are interesting for basic and applied research.

Feeding is, however, difficult to observe, as aphids do not move while inserting their stylets. A widely applied method to observe feeding is the electrical penetration graph (EPG) technique (McLean & Kinsey, 1964). By applying one wire on the aphid head and another one on a plant part, one can measure patterns in voltage changes. The analysis of these patterns is, however, onerous. Although a machine-learning algorithm has been devised recently for one species (Willett et al., 2016), studies on other species still rely on manual identification, which limits the application of the EPG technique. Another way to measure feeding is to observe aphid movement by automatic video tracking (Kloth et al., 2015). This promising approach requires however a video tracking platform able to discriminate small movements. This need for specialized equipment probably has prevented this method from being widely used so far. We thus conclude that feeding is difficult to observe directly.

Various attempts have been made to measure honeydew excretion as proxy for feeding. Honeydew drops can be directly counted (Schaefer, 1938), or collected e.g. on cardboard sheets with wax paper (Auclair, 1958) or chromatography paper (Kunkel & Hertel, 1976). But because the excreted honeydew is transparent, further staining with ninhydrin or silver nitrate may be needed (Kunkel & Hertel, 1976), a method that is described as extremely tedious (Paguia et al., 1980). Thus, an easily applicable method is needed to make either feeding or honeydew excretion more visible.

One notable technique that increased the visibility of feeding and honeydew excretion was the addition of neutral red as food colourant to a sugar solution (Mittler & Dadd, 1963). This method has recently been applied to study gene knockdown: Aphids were fed with a solution that contained dsRNA and neutral red or acridine orange as markers (Bilgi et al., 2017). Because the colouring stains the oesophagus and gut upon uptake, it was possible to separate aphids that fed on the diet from those which did not. This approach appears to be a promising tool to study insect resistance, although it would benefit from colourants that are readily ingested or cause lower mortality. Apart from this application, the idea of adding food colouring has been largely forgotten. Neutral red was originally used to quantify feeding on artificial diets, and to identify sugar as phagostimulatory substance (Mittler & Dadd, 1963). This finding and further studies led to the development of artificial diets, which are now available for several aphid species such as *Myzus persicae* (Dadd & Mittler, 1966) and *Acyrthosiphon pisum* (Auclair & Cartier, 1963; Febvay et al., 1988). Despite difficulties (van Emden, 2009), artificial diets have helped in studies of insecticide resistance (Sadeghi et al., 2009), insecticide uptake (Paula & Andow, 2016) and plant defence mechanisms (Cambier et al., 2001; Carrillo et al., 2011), but also unrelated topics such as diurnal rhythms (Joschinski et al., 2016) or alarm pheromone production (van Emden et al., 2014). The applicability of artificial diets might be further extended by adding colourants, but it is not known which colourants work in the considerably more complex diets. Here we report the use of colourings that reliably colour diets for *A. pisum* and other species. We test the toxicity of the colouring on an original pea aphid diet (Febvay et al., 1988) as well as on an updated recipe that incorporates newer dietary studies.

METHODS

Aphid rearing

We used a Medicago biotype of *Acyrthosiphon pisum* (clone LL01, kindly provided by Gerard Febvay), which was originally collected in the Lusignan area in France (Auclair, 1978). In addition, we used a red local clone of *A. pisum* (collected in 2013 from *Medicago sativa* near Würzburg, Germany), *Myzus persicae* (collected in a greenhouse from *Capsicum annuum*), *Macrosiphum euphorbiae* (collected in a greenhouse from *Lepidium spec*.) and *Aphis sedi* (collected from a wild growing *Sedum spec*.). Stock cultures of *A. pisum* were grown in low density on *Vicia faba*, and *A. sedi* was reared on the host *Sedum* plant from which it was collected. *M. persicae* and *M. euphorbiae* adults were kept on cut leaves of their host plant for one day before use. All plants and aphids were raised at 18°C under long-day conditions (16 h light: 8 h dark).

Diets and feeding technique

We used the artificial diet A0 by (Febvay et al., 1988) with a sugar concentration of 20% ("standard diet", Table VI.1). This recipe is nearly 30 years old and does not reflect newer studies on aphid nutrition, so we also tested a simplified recipe, in which we incorporated several improvements that have been reported for *A. pisum* and other species (Table VI.1). Specifically, we removed ornithine and ß-Alanine, riboflavin, cholesteryl benzoate, p-amino benzoic acid, biotine and pyridoxine. In addition, we replaced the metals by a single stock solution, and calcium citrate by ascorbic acid. To prepare 100 ml, 70 mg were allowed to react with the metal solution, whereas further 80 mg were directly added to the diet. Lastly, the order of the ingredients was adapted to that of van Emden & Harrington (2007).

To test food colourings, we fed both diets to *A. pisum* aphids. On the day before testing, we placed adult aphids on cut *V. faba* leaves to obtain newly born offspring. On the next day we removed the adults and reared the newly born aphid nymphs in petri dishes (35 mm diameter) without lid, which we sealed with Parafilm M (Bemis Company INC., USA). After adding approximately 300 µl of diet, we placed another layer of parafilm on top, thus squeezing the diet in between (Mittler & Dadd, 1963) and placed the petri dishes in climate chambers with 16:8 LD conditions at 18°C. The diet was replaced twice per week.

Table VI.1: Recipes for 100 ml standard diet and improved diet. Ingredients were added in the order of the list. Differences between diets are marked with superscripts. Except for ascorbic acid the ingredient concentrations do not differ between diets.

Standard diet		
	Ingredient	**(mg)**
	Sucrose	20 g
	Alanine	178.71
[1]	ß-Alanine	6.22
	Arginine	244.90
[2]	Asparagine H_2O	298.55
[2]	Aspartic acid	88.25
	Cysteine	29.59
	Glutamic acid	149.36
	Glutamine	445.61
	Glycine	166.56
	Histidine HCl H_2O	136.02
	Isoleucine	164.75
	Leucine	231.56
	Lysine HCl	351.09
	Methionine	72.35
[1]	Ornithine HCl	9.41
	Phenylalanine	294.53
	Proline	129.33
	Serine	124.28
	Threonine	127.16
[2]	Tryptophan	42.75
[2]	Tyrosine	38.63
	Valine	190.85
[1]	Calcium citrate	10.00
[1]	Cholesteryl benzoate	2.50
[2]	$MgSO_4$ $7H_2O$	242.00
[1]	p-Amino benzoic acid	10.00
[3,4]	L-Ascorbic acid	100.00
[1]	Biotin	0.10
[2]	Calcium panthothenate	5.00
[2]	Choline chloride	50.00
[2]	Folic acid	1.00
[2]	i-Inositol anhydrous	42.00
[2]	Nicotinamide	10.00
[1]	Pyridoxine HCl	2.50
[1]	Riboflavin	0.50
[2]	Thiamine HCl	2.50
[4]	$CuSO_4$ $5H_2O$	0.47
[4]	$FeCl_3$ $6H_2O$	4.45
[4]	$MnCl_2$ $4H_2O$	0.65
[4]	NaCl	2.54
[4]	ZnCl2	0.83
[2]	KH_2PO_4	250.00
	KOH	pH adjusted to 7.5

Improved diet		
	Ingredient	**(mg)**
	Sucrose	20 g
[2]	KH_2PO_4	250.00
[2]	$MgSO_4$ $7H_2O$	242.00
[2]	Tyrosine	38.63
[2]	Asparagine H_2O	298.55
[2]	Aspartic acid	88.25
[2]	Tryptophan	42.75
	Alanine	178.71
	Arginine	244.90
	Cysteine	29.59
	Glutamic acid	149.36
	Glutamine	445.61
	Glycine	166.56
	Histidine HCl H_2O	136.02
	Isoleucine	164.75
	Leucine	231.56
	Lysine HCl	351.09
	Methionine	72.35
	Phenylalanine	294.53
	Proline	129.33
	Serine	124.28
	Threonine	127.16
	Valine	190.85
[3]	L-Ascorbic acid	**80.00**
[2]	Thiamine HCl	2.50
[2]	Nicotinamide	10.00
[2]	Folic acid	1.00
[2]	Calcium panthothenate	5.00
[2]	i-Inositol anhydrous	42.00
[2]	Choline chloride	50.00
[4]	$CuSO_4$ $5H_2O$	0.47
[4]	$FeCl_3$ $6H_2O$	4.45
[4]	$MnCl_2$ $4H_2O$	0.65
[4]	NaCl	2.54
[4]	ZnCl2	0.83
[3,4]	L-Ascorbic acid	**70.00**
	KOH	pH adjusted to 7.5

[1] ingredient removed
[2] ingredient reordered
[3] We increased the concentration of ascorbic acid and used it as metal chelator. 80 mg were added directly, whereas 70 mg were allowed to react metal solution prior to use
[4]metals were dissolved in single non-concentrated solution and mixed with 70 mg ascorbic acid.

Screening for candidate colourants

We screened 32 different colourants systematically for their applicability in diets by feeding coloured standard diet to the clone LL01. Using a low sample size of only two petri dishes with ten newly born nymphs, we first tested each colourant in an undefined, low concentration. We examined aphids and their honeydew three to four days later, and recorded whether the colourant has precipitated out of the diet, how many aphids were still alive, and whether the honeydew was coloured. Colourants that dissolved and did not kill aphids, but also did not colour honeydew, were tested again in up to two higher, still undefined concentrations. This procedure inflates the rate of false negative samples, i.e. there might be more working dyes that we have missed due to low sample size or wrong concentrations. However, the procedure helped us select three candidate colourings quickly.

Toxicity tests on standard and improved diet

We measured the mortality of LL01 over 13 days to assess the toxicity of the most promising colourant, Brillant blue FCF (ready for use solution, 1.2% w/v with 85% purity; Ruth, Bochum, Germany, http://www.ruth-online.de/). We reared 50 newly born aphid nymphs, distributed over 5 petri dishes, on the standard diet, and 50 nymphs on coloured standard diet (0.8 mg/ml). We counted the number of dead aphids daily, and used the data for a survival analysis (Cox's proportional hazard model).

We then fed improved diet and coloured (0.65 mg/ml) improved diet in two petri dishes with 5 nymphs each. Additionally we tested the improved diet against the standard diet on a local clone on two petri dishes with 5 nymphs. We counted the number of dead aphids twice per week.

Because the concentrations of 0.8 mg/ml (standard diet) and 0.65 mg/ml (improved diet) caused high mortality after about one week (see results), we tested a lower concentration in a subsequent experiment. 30 aphids were reared individually on an improved diet with 0.125 mg/ml food colouring added. The aphids were observed for 13 days, with a single renewal of diet on day eight.

Tests on other species

We placed nymphs of the four species *A. pisum* (5 nymphs), *M. persicae* (5), *M. euphorbiae* (6) and *A. sedi* (7) in four petri dishes. We provided *M. euphorbiae* and *A. sedi* with standard diet plus 0.25 mg/ml Brilliant Blue FCF on the same day, whereas *A. pisum* and *M. persicae* developed on standard diet to L4 before being fed with the colourant. We monitored whether aphids and their honeydew were coloured.

RESULTS AND DISCUSSION

Simplified diet

Our method depends on an artificial diet, which was developed in the 1980's (Febvay et al., 1988). Since the development of this diet, various studies on aphid nutrition demonstrated that some components may not be needed. Indeed, a diet for *Myzus persicae* (Mittler & Dadd, 1962), invented at nearly the same time as the precursor of ours (Auclair & Cartier, 1963), evolved in a different direction and uses a slightly different set of ingredients. Because the preparation of the artificial diet is a time-consuming process, we suggest several improvements that reduce diet complexity (Table VI.1). We concentrated on ingredients that are only needed in low quantities (e.g. Biotin, Riboflavin) or are difficult to dissolve (cholesteryl benzoate) and thus slow the preparation of the diet.

Specifically, we removed ornithine and ß-Alanine, as these amino acids do not improve growth (Sasaki et al., 1991), and riboflavin, because it was proven harmful (Boisvert & Auclair, 1981). We additionally removed cholesteryl benzoate. *M. persicae* has been reared for 30 years without sterols (van Emden, 2009), and *A.pisum* can also survive over several generations on sterol- free diets (Akey & Beck, 1972; Bouvaine et al., 2012), potentially because they are provided by endosymbionts (Akey & Beck, 1972). Following this logic we removed biotine and pyridoxine, which are likely also provided by endosymbionts (Nakabachi & Ishikawa, 1999; Rao et al., 2015), and p-amino benzoic acid, which is not used in the *M. persicae* diet (van Emden & Harrington, 2007). Moreover, it was shown that higher levels of ascorbic acid are beneficial to aphid growth, especially if used as metal chelator (Mittler, 1976). We thus replaced calcium citrate and increased the concentration of ascorbic acid, simultaneously compensating for a loss by light degradation. Overall, we reduced the diet from 44 to 33 ingredients.

When we tested survival of clone LL01 on the improved diet, none of the 10 aphids died in 13 days (Figure VI.1). Similarly, a local clone suffered no casualties on standard and improved diet over 13 days, so we conclude that the changes in diet do not affect survival into adulthood. We did however not test whether the proposed changes reduce longevity or survival over several generations, as neither of the clones produces sufficient offspring on artificial diets.

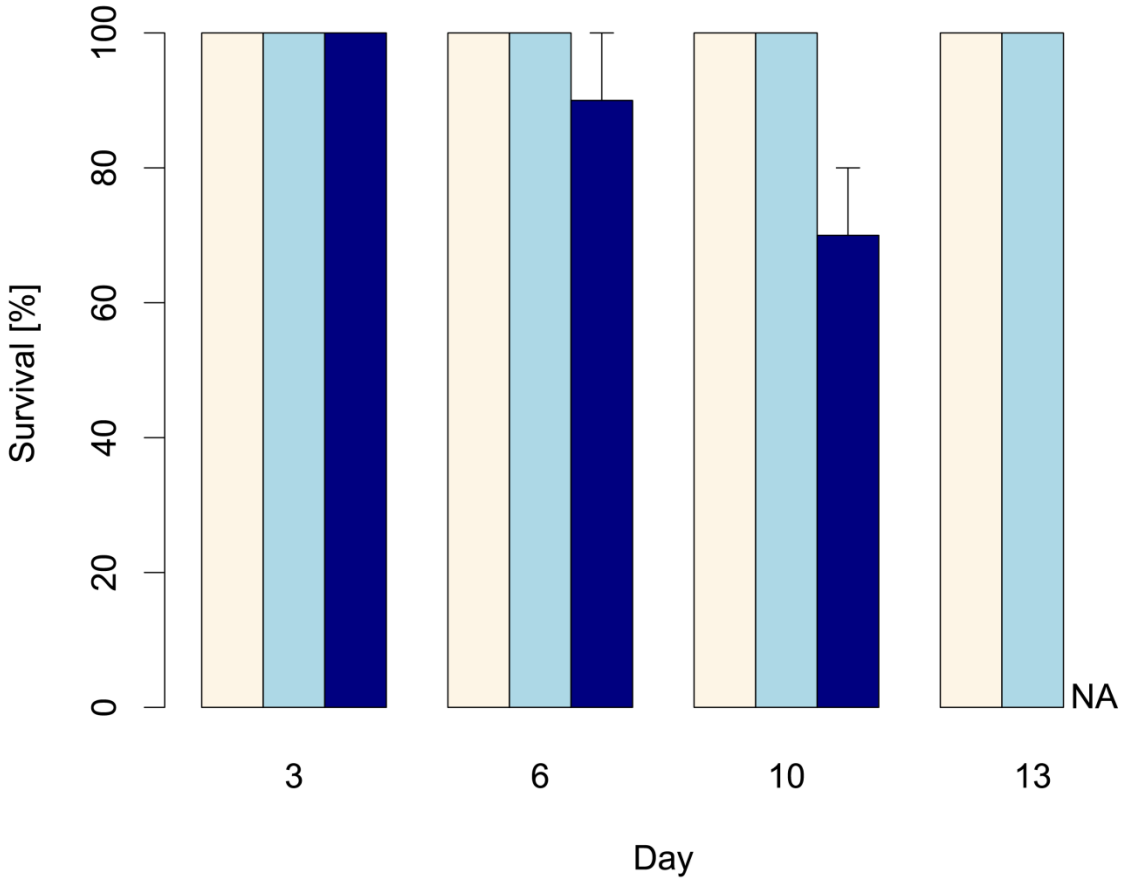


Figure VI.1: Survival of *A.pisum*, clone LL01, on improved artificial without colourant (white), with 0.125 mg/ml Brilliant Blue FCF (light blue) and with 0.65 mg/ml colourant (dark blue). 22 aphids were individually raised on diet with 0.125 mg/ml, whereas we fed 10 nymphs in 2 petri dishes in the other two treatments.

Screening for candidate colourants

We fed 32 candidate colourants in undefined concentration to *A.pisum* to follow nutrient uptake, and to obtain visibly stained honeydew (Table VI.2). Eleven colourants did not dissolve in sufficient amounts on standard diet, but 20 out of 21 remaining colourants were visibly ingested by at least some aphids, though ingestion was variable and in most cases only a small section of gut or oesophagus was coloured. We suspect that the aphids were probing in these cases, and did not ingest larger amounts of diet. Accordingly, 17 colourants caused high mortalities, so they were either toxic in the concentrations we used, or the aphids rejected the diet and starved. One further colourant stained neither the aphid gut nor the honeydew. The three remaining colourings Brilliant Blue FCF (Figure VI.2), acid fuchsine and blue dextran coloured the whole reproductive tract and honeydew. Acid fuchsine caused however some mortality within three days, and was also toxic to

M. persicae in another study (Bilgi et al., 2017). While blue dextran was not fatal, it provided only a relatively light staining. Brilliant Blue FCF on the other hand, provided strong colouring already in modest concentration, and did not cause high mortality within the first three days. Hence, we identified Brilliant Blue FCF as the most promising marker of aphid feeding.

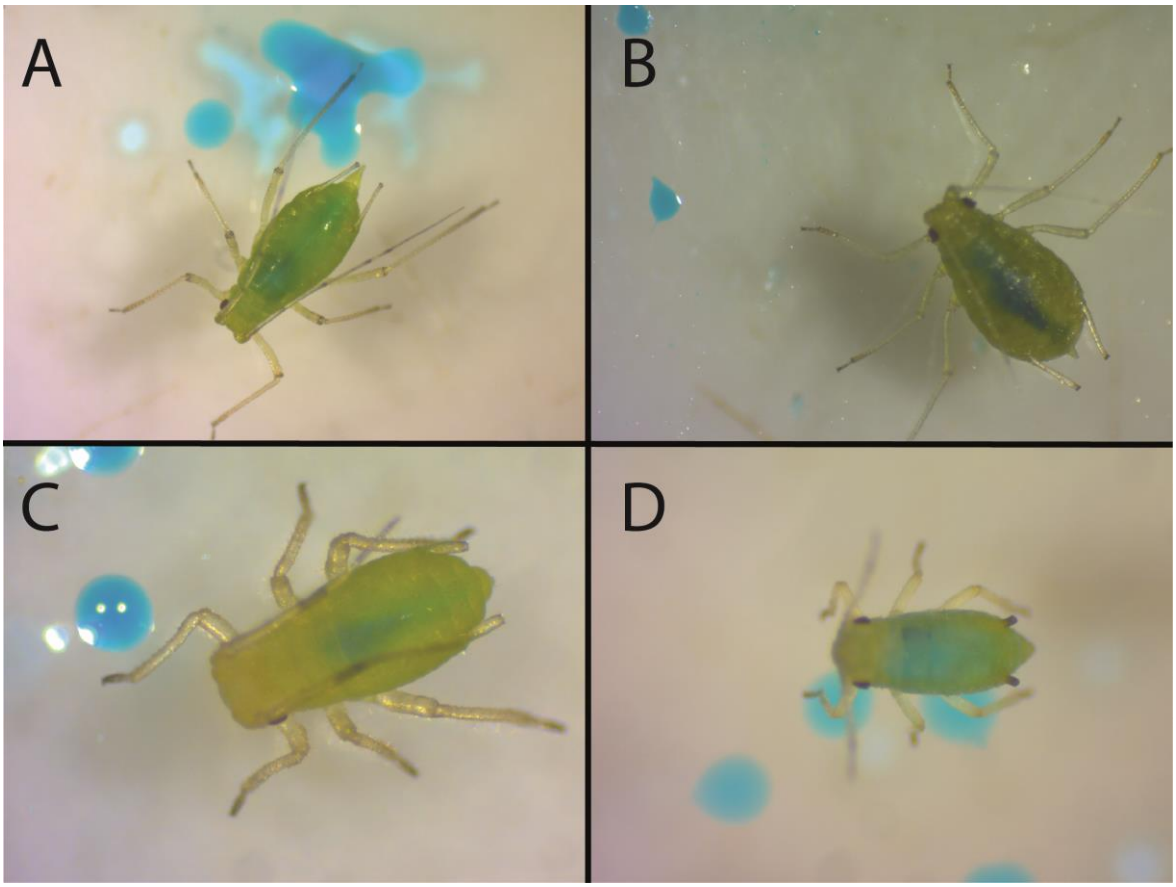


Figure VI.2: Four species fed with Brilliant Blue FCF (0.25 mg/ml in standard artificial diet). Panel A) *A. pisum*, B) *Myzus persicae*, C) *Macrosiphum euphorbiae*, D) *Aphis sedi*. All aphids were collected within 24 hours after being born. Six nymphs of *M. euphorbiae* and seven *A. sedi* nymphs were directly transferred to blue diet, whereas five *A. pisum* and *M. persicae* nymphs were reared on standard artificial diets first. In all aphids the blue food colouring reversibly stains the aphid gut until the diet is excreted as blue honeydew.

Table VI.2: Effects of the colourants on *Acyrthosiphon pisum*. All colours were tested on 10 nymphs on standard diet in undefined concentrations. Additives, which were negative but caused low mortality (mortality 1) were repeated in higher, still undefined concentration (mortality 2 and 3).

Colourant	company	solubility	petri dishes coloured honeydew	mortality 1	mortality 2	mortality 3
Brilliant Blue FCF	Ruth[1]	high	2	0%	0%	30%
Blue Dextran	Sigma	high	2	0%	0%	0%
Acid fuchsine	Waldeck	high	2	30%	10%	
Aniline Blue WS	Merck	high	2	100%		
Mucicarmine	Bayer	low	1	50%		
Borax carmine	Riedel-de Häen	high	1	100%		
Astra blue	Waldeck	high	0	0%	0%	
Rose Bengal	Waldeck	high	0	0%	100%	
Naphtol Blue Black	Aldrich	high	0	10%	100%	
Acridine orange	Ferak	high	0	80%		
Trypan blue	Merck	high	0	80%		
Azure II	Merck	high	0	90%		
Coomassie Brilliant Blue	Ferak	high	0	90%		
Toluidine blue	Merck	high	0	90%		
Bromocresol green	Riedel-de Häen	high	0	100%		
Bromophenol blue	Riedel-de Häen	high	0	100%		
Eosin Y	Merck	high	0	100%		
Janus green B	Merck	high	0	100%		
Light Green SF yellowish	Waldeck	high	0	100%		
Orange G	Waldeck	high	0	100%		
Safranin O	Merck	high	0	100%		
Celestine Blue	Chroma	medium	0	0%	40%	
Caerulein S	Kepec	medium	0	100%		
Basic fuchsine	Fluka	low	0	0%	100%	
Haematoxylin	Ferak	low	0	0%	100%	
Cresyl violet	Sigma	low	0	0%	0%	0%
Methyl orange	NA	low	0	0%		
Neutral red	Merck	low	0	0%	50%	60%
Nile blue	Aldrich	low	0	0%	0%	10%
Nuclear fast red	Merck	low	0	20%		
Bismarck brown	Kepec	low	0	50%	0%	50%
Carmine	Roth	low	0	90%		

[1] Bought as ready-made solution, 1.2% w/v with 85% purity; Ruth, Bochum, Germany,

Toxicity tests

We tested Brilliant Blue FCF in more detail on *A. pisum*. When we exposed 50 aphids to a concentration of 0.8 mg/ml, we found no effects on aphid health during the first three days (Figure VI.3). We observed that the aphids settled and fed on coloured diets as readily as on the standard diet, and the whole gut was coloured blue (cf. Figure VI.2). Hence, we conclude that in contrast to most other colourants, the aphids were not only probing on Brilliant Blue FCF, but used the coloured diet as food source. 80% of the aphids survived also the next three days, but then mortality increased ($z = 5.46$, $p < 0.001$), and the L4 nymphs did not moult into adults. In an improved diet with Brilliant Blue FCF added in high concentration (0.65 mg/ml), survival of 10 aphids decreased similarly to the standard diet (Figure VI.1). Hence the blue food colouring works equally well in the novel recipe, but very high concentrations deter feeding or affect the metabolism in both diets.

Because the high concentration caused mortality after one week, we reduced the colourant content to 0.125 mg/ml, as this proved sufficient to visibly colour gut and honeydew. Because initial tests did not show any evidence for elevated mortality, we used this low concentration in a subsequent experiment with 30 individuals (Figure VI.1). Experimental constraints did not allow exchanging the diet for one week, and eight replicates became contaminated after four days. All of the remaining 22 aphids survived for 13 days. We thus recommend a concentration of approximately 0.1 mg/ml for most applications, except where particularly strong colouring is required.

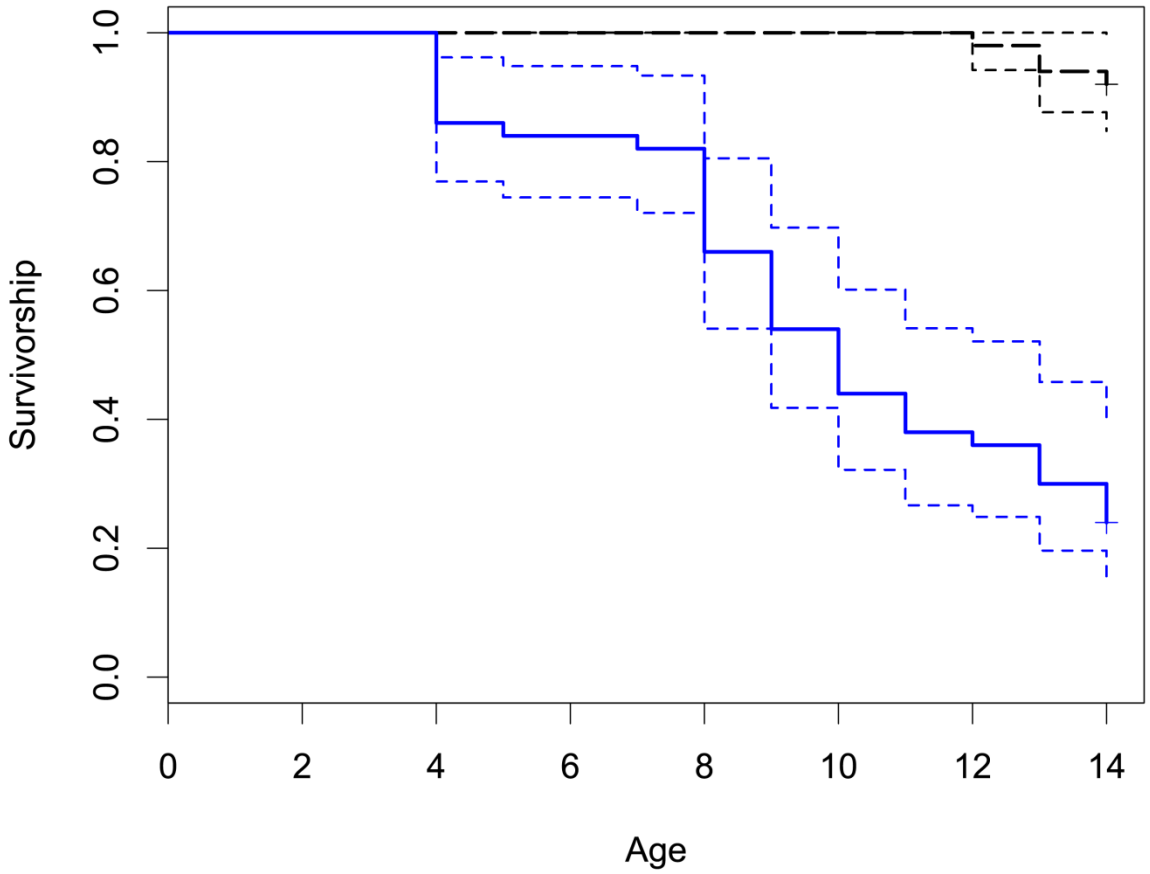


Figure VI.3: Survival analysis of *A. pisum*, clone LL01, on standard diet. We raised 50 aphids on diet with 0.8 mg/ml Brilliant Blue FCF added (blue, solid line), and compared survival to 50 aphids that were raised on standard diet without colourant (black, interrupted line). Dashed lines indicate confidence intervals.

Tests on other species

Another agricultural pest species that is frequently used as model aphid is *M. persicae* (e.g. Ramsey et al., 2007). Because *M. persicae* has been reared with success on artificial diets (van Emden, 2009), we tested whether Brilliant Blue FCF can also be applied to this species. We provide proof of principle, as the colour stained both *M. persicae* and the excreted honeydew in a single replicate with 5 aphids (Figure VI.2 B). Because the results are consistent with two more species we tested, *Aphis sedi* and *M. euphorbiae* (Figure VI.2 C, D), we suggest that the food colouring is useful for any species that can live on artificial diets. Artificial diets have been tested on various species, and polyphagous species were generally able to survive (Krieger, 1971; Wille & Hartman, 2008; Balvasi et al., 2009), whereas more specialized soldier-producing aphids have been difficult to rear (Shibao et al., 2002). We conclude that the colourant can likely be applied to a larger range of species, including several polyphagous pests.

Applications

Our study was motivated by the need to quantify aphid activity (Joschinski et al., 2016). We required a visible estimate of feeding to study diurnal rhythms, but we think that a method to make ingestion and excretion visible is relevant for many researchers. For example, Cambier et al. (2001) determined the toxicity of two plant metabolites on artificial diets. The metabolites appeared to be strong feeding deterrents, as indicated by restlessness of the aphids and lack of honeydew excretion. Brilliant Blue FCF could facilitate such studies by colouring the honeydew, and by making the amount of ingested diet visible. Another study needed to identify successfully feeding aphids to study gene knockdown by RNA interference (Bilgi et al., 2017). Neutral red and Acridin Orange were used to mark aphids that fed dsRNA in simple sugar solution. Although the authors succeeded with this approach, 20 % to 25 % of the aphids died within 24 hours, and only 25-30% were visibly coloured. These results are in line with our study, which found that aphids are either intoxicated, or reject these colourants after initial probing and starve. Thus Brilliant Blue might also be a better candidate to trace gene knock down. These examples show that studies which require evidence of aphid feeding could profit from using Brilliant Blue FCF. Currently this method describes diet ingestion qualitatively, though a scoring system of the coloring (Mittler & Dadd, 1963), or counting the amount of honeydew drops could yield semiquantitative results.

We see further application of the colourant beyond those of feeding and plant resistance. Neutral red was found to stain the salivary sheath (Mittler & Dadd, 1963), so it may be used to track the location of aphid feeding in studies of virus transmission. Furthermore, the colourant also stains the

honeydew. Hence, it might be possible to trace its fate, e.g. after being carried away by ants. We did, however, not test these potential applications so far.

Low concentrations of Brilliant Blue FCF did not interfere with feeding and are thus well suited as markers. In light of the observed dose-dependent mortality, we can however not exclude that higher concentrations cause long-term effects on survival or reproduction. We do not consider such limits as major drawbacks though, because we mainly aim to support studies with a marker for feeding and excretion, and these behaviours are on the scale of hours rather than weeks. Furthermore, the use of artificial diets already limits feeding and excretion studies to shorter time scales due to the adverse effects on aphid health (van Emden & Harrington, 2007). Thus, we conclude that concentrations of 0.1 mg/ml can be safely used in studies taking up to two weeks, but that higher concentrations are only advisable if the experiment is limited to less than three days.

CONCLUSION

We have shown that Brilliant Blue FCF as diet supplement can colour ingestion and excretion in *A.pisum* as well as in other species. The colourant is also compatible with an improved diet that reduces preparation time. Because higher concentrations increase aphid mortality, we recommend a concentration of 0.1 mg/ml for most purposes.

ACKNOWLEDGEMENTS

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One individual pea aphid, reared on the blue artificial diet. Two blue honeydew drops are visible in the background, and one colourless exuvia lies at the lower left.

CHAPTER VII CIRCADIAN CLOCK OF APHIDS

This chapter is an unpublished manuscript to be submitted in 2017 as:

Beer K*, Joschinski J*, Helfrich-Förster C' and Krauss J'. Working title: A damping circadian clock drives weak oscillations in metabolism and locomotor activity of aphids (*Acyrthosiphon pisum*). *,' : Equal contribution

SUMMARY

Aphids have a complicated seasonal life cycle that is driven by photoperiod (day length) measurements. It remains unclear whether photoperiodic induction relies on the circadian clock, a molecular mechanism that measures time of day. While aphids have traditionally been seen as hour glass models without circadian involvement, newer models explain photoperiodic induction with damped clock involvement.

To study clock output of *Acyrthosiphon pisum* aphids, we kept individuals on artificial diet in a novel monitoring setup and measured locomotor activity in light-dark (LD) illumination cycles of 16:08 hours and constant conditions (DD). In addition, we raised individual nymphs on colored diet to measure rhythms in metabolic activity. We found that *A. pisum* is day-active in LD, potentially with a bimodal distribution. In constant darkness rhythmicity of locomotor behavior persisted in some individuals, but patterns were mostly complex with several predominant periods. Metabolic activity, on the other hand, damped quickly. A damped circadian clock, potentially driven by multiple oscillator populations, is the most likely explanation of our results.

INTRODUCTION

The environment changes daily due to the earth's rotation around its own axis and the sun. In order to cope with these changes, organisms rely on an endogenous circadian clock which drives rhythms of approximately 24h in behavior and physiology (Allada & Chung, 2010; Mohawk et al., 2012). These rhythms persist even after removing all environmental time cues (Zeitgebers) like light or temperature (Roenneberg et al., 2003a). The internalization of cyclic patterns enables organisms not only to react to, but also to predict environmental oscillations, which may be advantageous in adaptation (Vaze & Sharma, 2013). Hence, circadian clocks play an important role in coping with diurnal change.

Circadian timekeeping might also be involved in the photoperiodic calendar and control of diapause induction. Bünning proposed that the circadian clock forms the basis of photoperiodism (Bünning, 1936). In contrast, Lees did not observe any circadian pattern in the photoperiodic reaction of aphids and excluded therefore the involvement of a circadian oscillator. He proposed an hourglass mechanism instead, in which some biochemical product accumulates during night, so that only sufficiently long nights can trigger a photoperiodic response (Lees, 1973; Lees, 1986). But some phenomena of his long night experiments cannot be explained by an hour glass mechanism, and a model with a damping circadian oscillator describes diapause induction better (Vaz Nunes & Hardie, 1993). By rephrasing the hour glass mechanism as a damping oscillator, the damped oscillator model unites the apparently contrary views on circadian (Saunders, 2005). However empirical data demonstrating the actual damping of circadian oscillations is largely lacking.

Aphids are in the center of many studies on photoperiodism. During summer the insect produces offspring via parthenogenesis which ensures quick population growth. In autumn sexual morphs are induced, which produce cold resisting eggs able to survive in winter (Simon et al., 2002). The switch in reproductive modes is induced by shortening day length (Marcovitch, 1923; Lees, 1959) and, to a lesser extent, drops in temperature. The extraordinary skill to adapt to environmental changes is probably one reason for the global distribution of aphids. Because various species are classified as crop pests (van Emden & Harrington, 2007), and aphid phenology (seasonal timing) is sensitive to climate change (Bell et al., 2015), there is also an applied perspective in studying aphid photoperiodism and its mechanisms. Due to both the history of research in photoperiodism and availability of long-term phenology data, aphids are well-suited to study the involvement of circadian oscillations in photoperiodism.

So far investigations on the circadian clock of aphids are few. Only one study identifies putative clock genes on the transcriptional level in the pea aphid *Acyrthosiphon pisum* (Harris) (Cortes et al., 2010). There are, however, behavioral studies that indicate a functional circadian clock in sexual as well as in parthenogenetic aphid morphs (Eisenbach & Mittler, 1980; Hodgson & Lane,

1981). One reason for this little number of studies on the aphid circadian clock could be the challenge to uncouple the aphid's activity from the plant's influence. Recently we described the first daily rhythms of aphids completely independent of their host plant (Joschinski et al., 2016), though rearing aphids in constant darkness has not been possible, making it difficult to investigate the clock.

In the present study we investigate two different outputs of the circadian clock, namely locomotor activity and metabolic activity independently of the host plant. We show that the aphid clock drives weak, but stable, circadian output rhythms in locomotion, whereas oscillations in metabolic activity dampen quickly in constant conditions.

MATERIAL AND METHODS

In order to investigate possible circadian output behavior of aphids, we performed two experiments: First, we assessed locomotor activity of aphids, using a novel method that allows constant monitoring of sap-sucking insects. Secondly, we tested for rhythms in feeding activity, by assessing excretion of colored honeydew drops. All experiments were performed independently of the host plant on an artificial diet (based on Febvay et al., 1988; 20% (w/v) sugar). For the metabolic activity experiments we reduced the diet by eight ingredients, reordered the ingredients list, and supplemented it with 1.25 mg/ml Brilliant blue FCF (chapter VI).

We reared individual aphids either in petri dishes (⌀ 35 mm) or in custom-made monitors (see section "locomotor activity"). In both cases, we closed the aphid containment with parafilm M (BEMIS COMPANY INC., USA), added 500 µl artificial diet, and sealed the other side with another layer of parafilm (Mittler & Dadd, 1963). The aphids could thus access the diet from below by piercing the parafilm and inserting the stylets into the food source. The artificial diet was sterile filtrated through a 0.45 µm Minisart® syringe filter (Sartorius, Germany), and all materials that came in contact with the diet were sterilized before use.

For all experiments we used an asexual *Acyrthosiphon pisum* line (LL01), a green alfalfa biotype from the Lusignan area that was kindly provided by G. Febvay (INRA Lyon, France). Stock cultures were kept on Pisum sativum (L.) var. Fuego plants in climate chambers (Sanyo/Panasonic MLR-H series; 18 ± 0.5°C, 80% ± 10% RH, LD 16:08). All statistical tests were performed in R version 3.1.1 (R Core Team, 2014)

Locomotor activity:

For the locomotor activity experiments we placed adult aphids, which were raised on plants, in a petri dish with artificial diet for one day. We used the one day old offspring of these aphids in our experiments in order to control for age. We adapted the DAM2 (Drosophila Activity Monitor) (Trikinetics, USA), which monitors locomotor activity via an infrared-light barrier (IR-beam), to aphid specific requirements. We placed 500 µl artificial diet in a shortened micropipette tip (volume 1000 µl) sealed with Parafilm, so that the aphids were provided with food *ad libitum* during the whole experiment. The tip was then placed on top of the monitor tube (⌀ 7 mm) while the activity monitors were placed horizontally (Fig. VII.1A). The insects were illuminated from the sides and not from above during the light phase. In order to detect as much activity as possible we limited the animal's roaming space to 1 cm and positioned the IR-beam directly under the micropipette tip to detect aphids moving on the food source (Fig. VII.1A close up picture). The setup allowed monitoring 32 aphids simultaneously. All animals were entrained as nymph to an LD 16:08 regime (16 hours light and 8 hours darkness) for at least 12 days. A light intensity of 200-400 lux was produced by white light LEDs (depending on the position of the monitor tube) in the light phase and 0 lux in the dark phase while temperature and humidity were kept constant (18 ± 0.5 °C, 80% ± 10% RH) in the incubator (Percival INTELLUS, CLF Plant Climatics GmbH, 86637 Wertingen, Germany). On day 13 one treatment group (32 aphids) was released into constant DD conditions whereas another group of 32 aphids received LD conditions throughout their life.

Activity data was recorded in beam crosses per minute and evaluated with the ImageJ software plugin ActogramJ (Schmid et al., 2011) (Fiji ImageJ Version 1.49, © Wayne Rasband, National Institutes of Health, USA). We tested for rhythmicity in activity of adult aphids across at least five consecutive days in entraining (LD) and free-running (DD) conditions with Lomb-Scargle (LS) and chi-square periodogram (CS) analysis (period 1140 - 1740 minutes; smoothing factor 10 (only in chi-square method); p-level 0.05). The free running period (FRP) of the endogenous clock was depicted only in periodograms with bouts clearly above the significance level, whereby many individuals showed complex rhythms with more than one significant predominant period in the periodogram analysis. Individuals that did not undergo all four moltings were excluded. In case of several small spikes rising over the significance level, we appointed the periodogram as false positive and the animals as arrhythmic. Differences in the groups "arrhythmic", "rhythmic" (with simple circadian rhythms) and "complex" (with complex rhythms) between LD and DD conditions were tested with Fisher's exact test and Pearson's Chi-squared test with Yates' continuity correction. We calculated the average day activity profile and mean activity in light and dark phase with the activity data of several consecutive days in Microsoft Excel (2013 Microsoft Office) and day activity was tested with Wilcoxon rank sum test. We did not calculate average days and mean

activity of day 8-12, because former studies have shown that in this time the aphids undergo their last molting with individual variation. During this period we could not generally appoint the aphids to either nymph or adult status. All statistical tests were performed in R version 3.1.1 (R Core Team, 2014).

Metabolic activity:

We measured metabolic activity simultaneously under LD 16:08 and constant darkness for eight days. We conducted the experiment in climate chambers (Sanyo MLR-352H) at 18°C and 70% humidity under a 15000 lux fluorescent light source.

We placed 100 adult aphids on freshly cut broad bean leaves. On the next day, we collected 120 nymphs and discarded the adults. We placed the nymphs individually in petri dishes and fed each with 500 µl colored artificial diet. 60 aphids were moved into DD after five hours (Zeitgeber time (ZT) 12 = 12 hours after lights on), whereas the other 60 nymphs remained in LD. At ZT 21 (i.e., three hours before lights-on), we counted all exuviae and marked all honeydew drops which have been produced so far. We then counted honeydew and exuviae every 3 hours (8 measurements per day).

Four observers have been involved in taking measurements. The time schedule was allocated in a non-random order, so that the two measurements per day were 12 hours apart, and that each observer occupied a different time slot every day. Measurements started at ZT0 for the first 30 Individuals from LD, at ZT 0.5 for the first 30 individuals from DD, ZT1 for the other half in LD, and ZT 1.5 for the other half in DD. We took care to always measure the replicates in the same order, and within the same 30 minutes block. Measurements in the light phase were conducted under room light while measurements in the dark phase were made under red LED light. The excreted blue honeydew drops were equally visible under both light conditions, as evidenced by a control in which we counted 10 replicates from the dark treatment again under lights-on and found no difference in drop numbers.

The first half of the replicates in the LD treatment was removed from the experiment, because they were accidentally taken into room-light during lights-out in the first night. Hence, sample sizes were 30 in LD and 60 in DD. Because we did not renew the diet during the experiment in order to reduce disturbance, petri dishes became contaminated during the experiment and were subsequently removed. Thus, sample sizes decreased over time, with 80% of the samples remaining for at least 5 days. In LD sample size decreased in total from 30 to 14, and in DD from 60 to 17.

Molting occurred three times per individual, i.e. aphids were in the final larval stage at the end of the experiment. To test for rhythmicity in molting, we applied a generalized linear mixed-effects model with binomial error distribution (Bates et al., 2015) with the fixed factors “time of day” and “day”, and with the random term (“time of day” | “ID”). To test whether molting individuals produce less honeydew, we pooled all time points and applied a chi-square test of goodness of fit (2x2 contingency table: drop production vs molting). Because molting individuals indeed produced less honeydew (see results), we used a reduced dataset with only non-molting individuals (6% of all measurements removed) to test for rhythmicity in honeydew production. We also validated all models with the full dataset.

Only 3.4 % of the aphids produced more than one honeydew drop in the observed time interval of three hours, so we treated the response as binomial. We modelled honeydew production in the LD-treatment like molting, with a generalized linear mixed-effects model that includes “time of day”, “day” and the random term (“time of day” | “ID”). For the DD treatment, we expected the effect to dampen over the course of the experiment, so we incorporated a change in effect size over time. Hence, we used the fixed factor “time of day”, interacting with the continuous variable “time since start”, and the random term (“time of day” | ID). Because p-values are not reliable for GLMMs (Halekoh & Højsgaard, 2014), we report only confidence intervals (library "effects", Fox, 2003).

RESULTS

Locomotor activity: circadian rhythms in LD and complex rhythms in DD

We monitored locomotor activity rhythms of aphids during their whole live from nymphal stage L1 onward. When we compared the activity of nymphs (age: 1-7 days) to adult aphids (age: 13-23 days), both the young aphids (Fig. VII.1B) and the adult aphids (Fig. VII.1C) showed significantly higher average activity during the light phase ($p(\text{nymphs}) < 0.05$, $p(\text{adult}) < 0.05$, paired Wilcoxon signed rank test). The activity patterns of the aphids were, however, disrupted by activity breaks of entire days during the first 9 days of their life (Fig. VII.2A/B), leading to lower activity levels in nymphs (0.08 ± 0.02 counts/min) than in adult aphids (0.31 ± 0.07 counts/min). The adults displayed two higher activity bouts in the average day, one after switching on the light and one in the late light phase, and activity levels in between were lower.

While we detected diurnal rhythms in activity of adult aphids in LD, the average actogram in constant conditions was arrhythmic over a measurement period of at least 19 days in the set up (Fig. VII.2A/B). In the individual analysis we found that many of the adult subjects (67 %) showed no activity at all during the first three days after switching to constant conditions of DD at day 13 (Fig. VII.2B). We were able to monitor activity for up to 29 days and determined the percentage of rhythmic individuals in LD and DD conditions via periodogram analysis (Lomb-Scargle (LS)

method in Fig. VII.2C and chi-square periodogram (CS) method in Fig. VII.2D) for activity periods of at least 5 continuous days. In DD (N = 15) 13 % (CS: 0 %) of the aphids were rhythmic and 80 % (CS 53 %) displayed complex rhythms while in LD (N = 15) 60 % (67 % with CS method) were rhythmic and 33 % showed complex rhythmicity.

In both periodogram analysis methods the difference in rhythmic individuals between light treatments was significant (LS: $p<0.05$; CS: $p<0.01$, Fisher's exact test). In a comparison of the two rhythm types (simple circadian and complex rhythmic), the percentage of complex rhythms was significantly higher under DD with LS analysis ($\chi 2=5.4$, $p<0.05$). The CS analysis yielded no significant difference ($p<0.5$, but expectation value<5), possibly due to the higher percentage ($p<0.01$, Fisher's exact test) of individuals classified as arrhythmic in DD conditions.

Metabolic activity: Bimodal rhythmicity profile under LD and DD

We detected no rhythmicity in molting in either treatment (supp. Fig. VII.1). Molting suppressed honeydew excretion, because only 13.75% of the molting individuals in LD produced honeydew drops, compared to 33.76% of the non-molting individuals ($\chi^2 = 13.75$, $p < 0.001$). In DD the ratios were 18.13% vs. 29.41% ($\chi^2 = 9.38$, $p < 0.01$).

During the non-molting time points, the aphids produced on average 2.6 drops per day in LD and 2.4 drops per day in DD. In contrast to molting, we found a marked rhythm in honeydew production under LD conditions (Fig. VII.3A/B). The activity profile peaked during day-time, and was apparently bimodal, though the confidence intervals overlap (ZT 21: 0.17 - 0.31 drops; ZT 9: 0.37 - 0.54 drops; Fig. VII.3B). Under constant darkness (Fig. VII.3C/D) the only discernible peak at ZT 12 (0.51-0.75 drops) damped quickly, being visible for only the first two days.

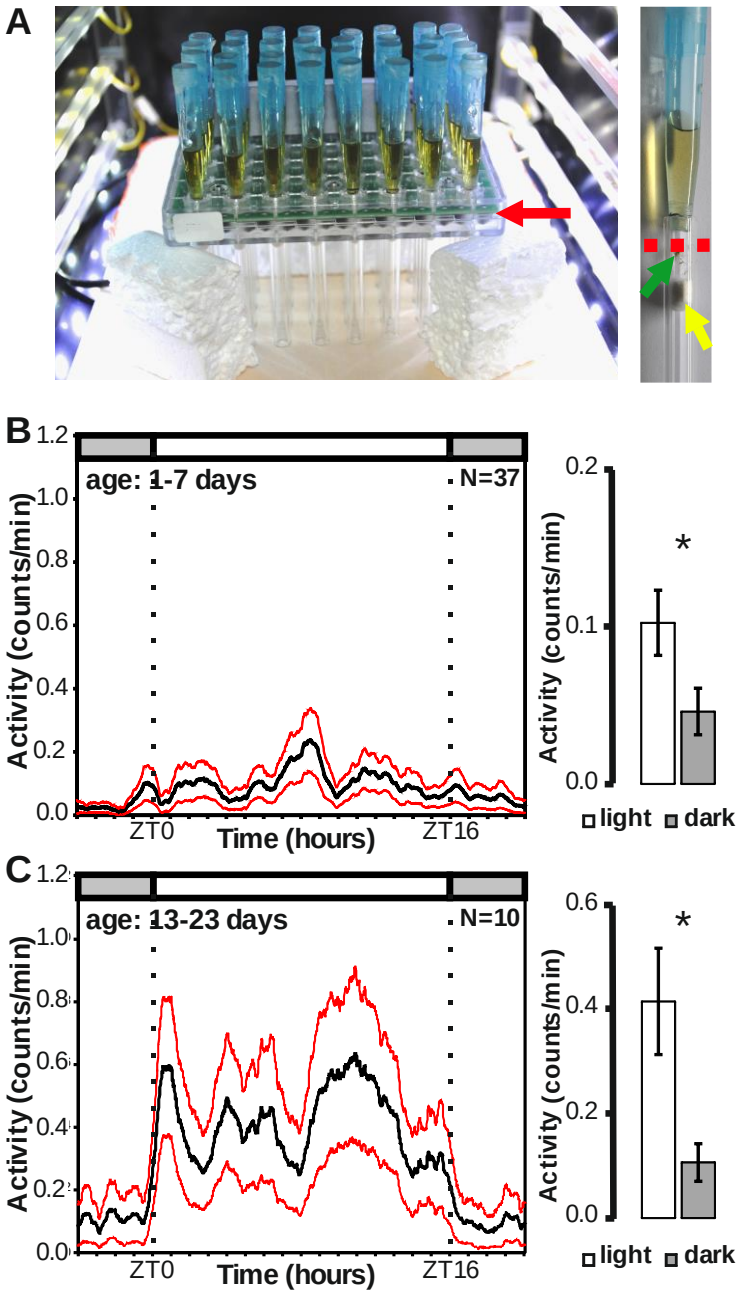


Figure VII.1: Locomotor activity monitoring set up and diurnal activity profile in aphids.
A: monitor(DAM2, Trikinetics) placed horizontally with illumination from the sides. 32 IR-beams on the monitor detect movements of individual aphids in glass tubes (close up picture) with blue food tubes on top. Aphid (green arrow) in a monitoring glass tube hanging from the food tube. The roaming space was limited by a cotton plug (yellow arrow). IR-beam (red arrow and dotted line) is positioned on the mid plane of the monitor and detects moving aphids.
Average daily activity profile of nymphs **(B)** and adult **(C)** aphids measured by counting IR-beam passages (black line = mean activity, red line = standard error). Average day is calculated for day 1-7 for nymphs and day 13-23 for adult aphids. The light regime (LD 16:08) is indicated by the white (light phase) and grey (dark phase) bars above the graphs. **Panels on the right**: Mean activity of nymphs (1-7 days, B) and adult aphids (13-23 days, C) during light and dark phase.

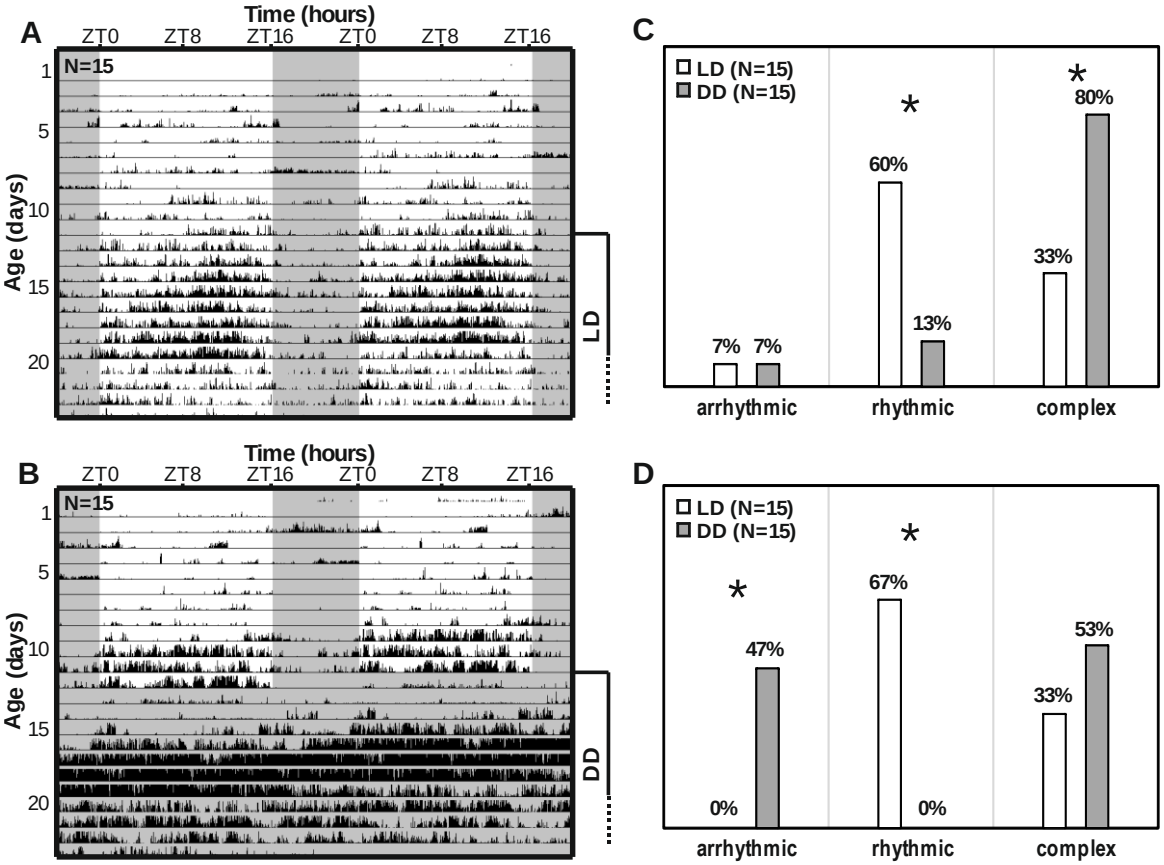


Figure VII.2: Locomotor activity rhythms of aphids under LD cycles and constant conditions. **A:** Aphid activity plotted in average actograms (double plots of two consecutive days). Animals are monitored from nymphal stage L1 onwards for several days in LD 16:08. Light regime is indicated by the white (light phase) and grey (dark phase) background in the actograms. Activity levels day 20-23 are lower because a few aphids died in this period (in A: 4, in B: 1)
B: Like in A for the first 12 days in the setup, then aphids are released into constant darkness (DD). Percentage of rhythmic individuals analyzed from day 13 onward for at least 5 consecutive days with activity (marked in A and B) in periodogram analysis with either Lomb-Scargle **(C)** or Chi-Square **(D)** method of testing rhythmicity.

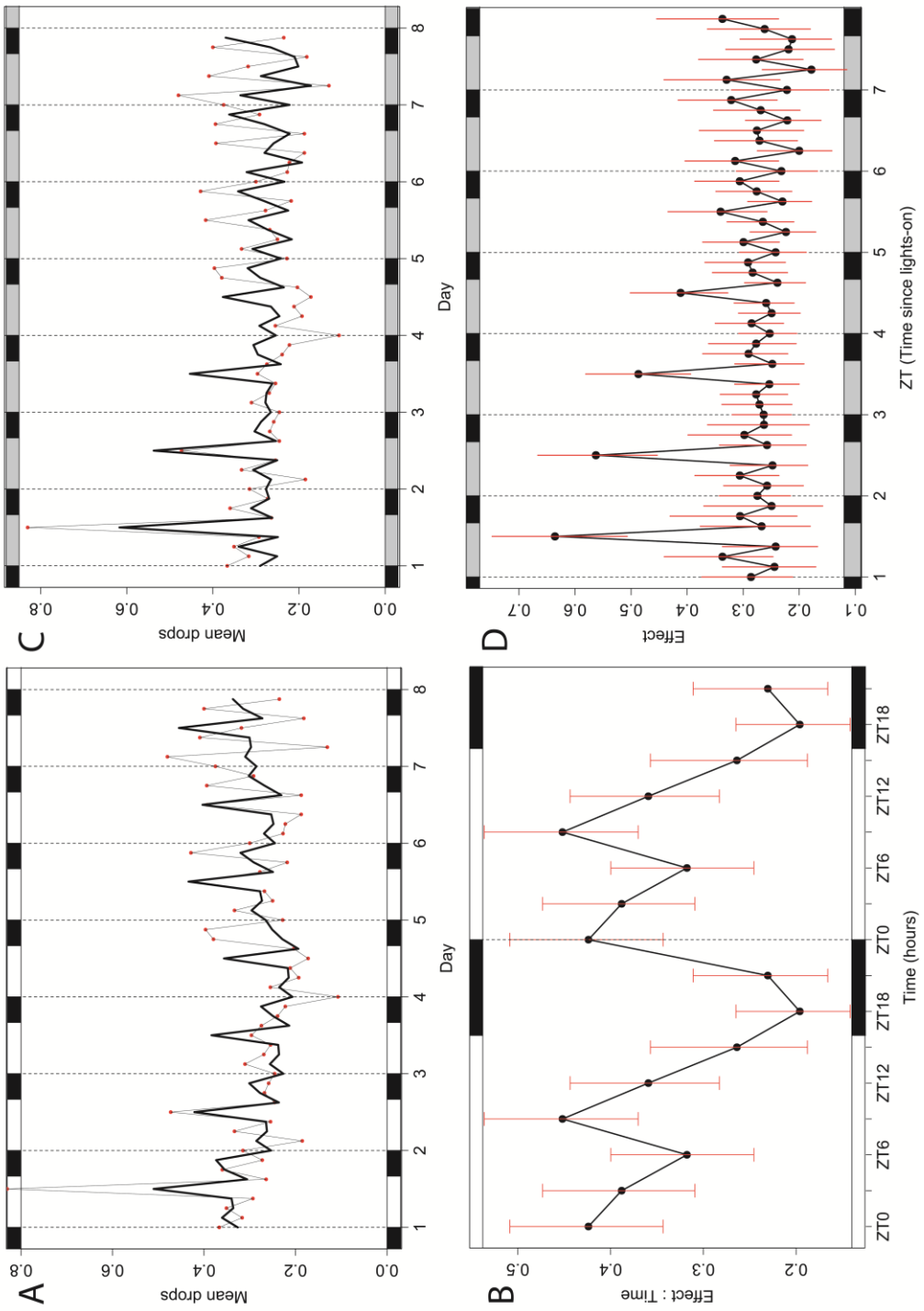


Fig. VII.3: Metabolic rhythms, measured as honeydew excretion, under LD and constant conditions. A. Mean honeydew excretion of 30 individually reared aphids (red dots, connected with grey lines) under 16:08 LD conditions. The rhythmicity was modelled with a binomial GLMM (thick black line). B. Effect plot of "time of day". Red bars indicate confidence intervals. Data is double-plotted for clarity. C Mean honeydew excretion of 60 individually reared aphids (red dots) under DD, and model output (thick black lines). The model includes a "time of day" : "time since start" interaction, i.e., a rhythmic pattern that damps over time. D Effect plot of the damping component. Red lines indicate confidence intervals.

DISCUSSION

By combining measurements of aphid locomotor activity and honeydew production, we find that aphid activity is bimodal under LD conditions. In constant darkness the rhythm persists initially, but then dampens quickly and individuals display complex rhythmicity.

Molting

Molting did not appear to be rhythmic. However, in contrast to the colored honeydew drops the white exuviae were very difficult to detect under dim red light conditions, which potentially increased variance in the measurements. Further studies will need to verify that molting is indeed not rhythmic. We decided to measure molting mainly because it causes aphids to stop moving for longer time periods (Auclair, 1959), and thus disrupts the activity in the young aphids. By removing molting individuals from the analysis of metabolism, we corrected for these gaps in activity. The disruption by molting was more severe in our measures of locomotor activity, where we did not obtain simultaneous observations of molting on the same individuals. Therefore we considered metabolic activity of nymphs, but locomotor activity of adults for analysis of circadian rhythmicity.

Generally low activity levels

We adapted a commercially available measurement system for locomotor activity of flies to monitor aphid rhythms with high data throughput. This novel monitoring method provides the first measurements of aphid locomotor activity rhythms independent of the host plant. It was sensitive enough to monitor adult aphids as well as nymphs, though the general activity level was lower than in most other insects. For example *Drosophila melanogaster* (Menegazzi et al., 2012), *Musca domestica* (McCarthy et al., 2015) and *Apis mellifera* (Beer et al., 2016) are approximately 10 times more active than the aphids in our locomotion setup. This finding can partly be explained by the unusual food source, which is known to compromise performance (van Emden & Harrington, 2007). The aphids produced on average 2.5 honeydew drops per day, which is lower than the 4-8 drops/day reported in studies on plants (Auclair, 1958). On the other hand, different linden bug (*Pyrrhocoris apterous*) strains, which are of the same order (Hemiptera) and also feed by sucking, show similarly low activity levels (Pivarciova et al., 2016). Thus, the low locomotor activity levels might be related to the sap-sucking feeding behavior.

Aphid activity with a bimodal pattern in LD

Despite the low activity levels, aphids were clearly day active in both locomotion and metabolism. These results are in line with various studies that detected diurnal rhythms on plants (Hodgson & Lane, 1981; Gomez et al., 2006; Taylor et al., 2012) as well as independently of the host plant (Joschinski et al., 2016). Furthermore, aphids were shown to find their host plants more effectively in day light conditions (Hardie, 1989; Narayandas & Alyokhin, 2006), indicating adaptation to a diurnal lifestyle.

The activity was bimodal in both locomotor and metabolic activity with one peak around the time of lights-on and another one in the late light phase. Although bimodality was in locomotion and metabolism independently not significant, the shared time profile is certainly suggestive. Bimodal patterns of activity were described in a variety of different animals, for example fruit flies (Helfrich-Förster, 2000), mosquitoes (Clopton, 1984), birds and rodents (Aschoff, 1966), and have been linked to multi-oscillator systems (Saunders, 2002).

The aphid clock is a damping circadian oscillator

While aphids showed clearly rhythmic behavior in LD, the patterns in DD were more complex. The majority of individuals in the locomotor assay were not active at all in the first few days in constant conditions and a damping rhythm would be hard to observe in this case. This lack of activity after switching to DD has been observed before in *D. montana* (Kauranen et al., 2012) and the authors argue that this inactive period might have increased the number of arrhythmic test subjects. Indeed we find a higher percentage of arrhythmic individuals in our DD experiments with the CS analysis method. We would like to concentrate our attention to the LS method, because this method detects rhythms more efficiently with low signal to noise ratio and significance levels in the CS method can be incorrect, if less than 10 days are analyzed (Ruf, 1999; Refinetti et al., 2007). Nevertheless in our experiment we see overall similar results with the two analysis methods. After the first few days without locomotor activity, the majority (LS: 93 %, CS: 53 %) was still rhythmic (either simple circadian or complex rhythms) in constant conditions, indicating that the behavior is governed by the circadian clock. However, complex rhythms with 86 % (LS) and respectively 100 % (CS) dominated the comparison of different rhythms types. In contrast, only 35 % (LS) and 33 % (CS) of detected rhythms were complex in LD.

The results from metabolic activity are slightly different: There is only one activity peak left in DD, which is probably due to a melting of the separate peaks like it has been observed in Drosophila melanogaster activity (Helfrich-Förster, 2000). This peak damped quickly during the first 3-4 days in constant conditions. The observed damping of circadian rhythms could either be due to desynchronization of individual persisting rhythms or damping of individual rhythms, but

the low number of honeydew drops and the resulting binomial data make it difficult to assess damping on the individual level. We thus incorporated inter-individual variation in a mixed-effects model to exclude population-level damping. The model is constrained in that the period length is fixed at 24h, but we find it unlikely to affect our conclusions, given the 3-hour intervals of the data.

At the moment we can only speculate about the mechanisms that give rise to complex rhythms and individual level damping in DD. Complex rhythms are not unusual, and have been described before for example in mosquitoes (Clopton, 1984) and New Zealand Weta (Lewis et al., 1991). Lewis and coauthors proposed a two population model of circadian oscillators, driving different output rhythms. In this model different clock neuron populations in the oscillator are weakly coupled and oscillations driven by them become asynchronous in DD. Therefore different FRPs for the oscillator subgroups are detected, which creates complex rhythms as well as a damping in one circadian output rhythm, and eventually arrhythmicity on the individual and/or population level. We find this model appealing for aphid clocks, because it simultaneously explains the damping in one output and complex rhythms in the other output. Moreover, the model provides an explanation why some studies found clear circadian patterns (Eisenbach & Mittler, 1980), whereas other studies with different outputs indicate hour glass mechanisms (Lees, 1959). Further studies on the molecular organization of the clock are needed to verify this model.
Moreover, since aphids produce honeydew during feeding and therefore metabolic and locomotor activity is directly connected, it is likely that similar mechanisms operate for these two outputs. Like the complex rhythms in locomotor activity might lead to dampened rhythmicity of the individual, damping of metabolic activity could occur at the individual level. Overall we found complex circadian rhythms in locomotion and quick damping in metabolism, and we argue that the aphid circadian clocks cannot drive strong activity rhythms.

The damping circadian oscillator model

Aphids played a central role in the development of the hourglass mechanism of the endogenous clock, which Lees postulated for the diapause induction of aphids (Lees, 1973; Lees, 1986). Lees found no circadian involvement in diapause induction, causing him to question the coincidence models.
However, various aphid behaviors were found to be controlled by a circadian oscillator. For example clock gene homologues have recently been characterized (Cortes et al., 2010), and their mRNA was shown to cycle. On the behavioral level the circadian clock was shown to govern the release of aphid sex pheromone (Eisenbach & Mittler, 1980), and fresh weight-gain and larviposition (Hodgson & Lane, 1981). In the latter experiment, aphids were disrupted by shifting

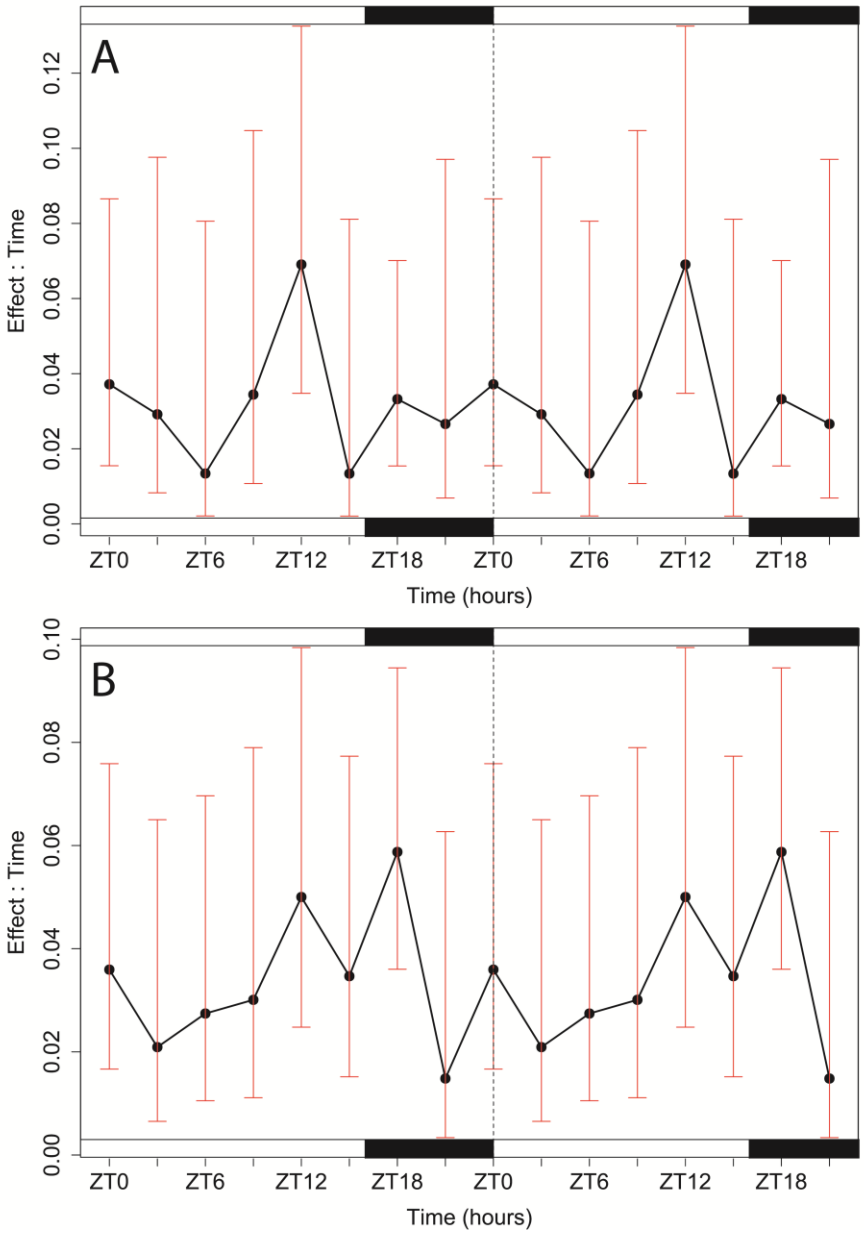
the light phase and the rhythms needed more than 3-4 days to re-entrain to the new conditions, which led the authors to the conclusion that this behavior is governed by a circadian oscillator.
As an alternative model to the hourglass model, the circadian oscillator model with damping rhythms of Vaz Nunes & Hardie (1987) can simulate the "hourglass reaction" observed in diapause induction of aphids. However, although this model fits to the data of Lees, empirical data showing actual damping of rhythms is missing. Our study supports the model of an oscillator driving damping circadian rhythms in two points:
Firstly we observe circadian oscillations in behavior and metabolic activity in DD, which would not be the case if the aphid endogenous clock would have an hourglass mechanism. Secondly, we see a clear damping of activity rhythms in the first few days in constant conditions, at least in metabolic activity. Hence, our study provides a first empirical evidence for the damping oscillator model.

Oscillators driving weak rhythms: adaptation to seasonal changes?

Though anticipating daily reoccurring events is an advantage, an oscillator that drives strong circadian rhythms over a long time is potentially less flexible in resetting to seasonal changes. There are environmental situations on earth that require organisms to adapt to extreme deviations from diurnal rhythms. For example reindeer living in Polar Regions have been found to be arrhythmic during extreme illumination conditions of the arctic summer and winter (Lu et al., 2010). This adaptation enables those animals to optimally use the short season for gaining resources and hence show a strong seasonal rhythm in locomotor and metabolic activity. A similar seasonal adaptation of the circadian system has been found in Drosophila species, *D. montana* and *D. ezoana*, living in Northern Europe (Kauranen et al., 2012; Vaze & Helfrich-Förster, 2016). Unlike *D. melanogaster*, which reacts only to diapause cues, these flies are exposed to extreme photoperiods and evolved a very robust diapause induction, while they have poor circadian activity rhythms in constant conditions. We can certainly draw here parallels to our results on aphid rhythmicity. Like the polar reindeer or the Northern *Drosophila* species aphids exhibit very weak circadian rhythms, while the seasonal response to shortening photoperiod is highly robust.
The weak circadian response in aphids might be the reason why most interest so far was put on the investigation of photoperiodism while the circadian clock remained widely unattended. But this way a complete picture of clock output characteristics was missing. Our study demonstrates that the investigation of different outputs, by combining expert knowledge of different disciplines is important to gain a better knowledge about special properties of the aphid circadian clock. There is an ongoing discussion if the circadian clock and the annual photoperiodic clock are two independent timing systems or if they are at least partially the same (Danks, 2005; Kostal, 2011).

Further investigation, also on the molecular level, of the circadian clock in aphids might provide us with a deeper understanding of the potential involvement of the circadian system in seasonal timing.

SUPPLEMENTARY MATERIAL



Supp. Fig. VII.1: Double-plotted effect plot for molting rhythms of 30 individual aphids in LD (panel A) and of 60 individual aphids in DD (panel B). Molting occurred three times per individual.

Sexul form of the pea aphid. The white eggs are visible through the cuticula of the aphid.

Comparison of parthenogenetic embryos (lower left) and eggs (right), dissected from parthenogenetic and sexual adults.

CHAPTER VIII: GENERAL DISCUSSION

Climate change is amongst the most pressing challenges of the 21st century (IPCC, 2014). Many taxa already responded visibly to climate change and advanced their phenology (Parmesan & Yohe, 2003), but rates of phenological adaptation differ among species and trophic levels (Thackeray et al., 2016). This loss of synchrony among species causes fitness costs (Visser et al., 1998) and affects community composition (CaraDonna et al., 2014), thus there is an urgent need to understand why species vary in their rates of phenological change. Links between constraints of phenology shifts and their mechanistic basis are, however, rarely studied, because biological disciplines are little connected (Visser et al., 2010), and the underlying time-keeping mechanisms are frequently overlooked (Helm et al., 2013). Moreover, the physiology and molecular biology of time-keeping mechanisms is mostly studied in laboratory environments (Marques & Waterhouse, 2004; but see Helm & Visser, 2010; Kronfeld-Schor et al., 2013) on few model organisms and in isolation from other species. Hence the natural variation of life-history strategies is ignored (Bradshaw & Holzapfel, 2010). As part of the collaborative research center "Insect timing", I studied one constraint of phenological adaptation (day length) on two trophic levels, and linked it to the diurnal niches of the organisms. I then focused on the circadian clock and its connection to the diurnal niche as well as to day length measuring and species phenology.

Day length constraints of *Acyrthosiphon pisum*

Acyrthosiphon pisum is sensitive to climate change, as the phenology shifted over the last 50 years to an earlier season start in spring, and later season end in autumn (Bell et al., 2015). Parthenogenetic forms may in the future even persist over the whole year, because a warmer environment can cause aphids to lose the sexual cycle (Simon et al., 2002). In addition, aphids can disperse over long distances (van Emden & Harrington, 2007), so range expansions to different latitudes can be expected with climate change. Overall, the seasonal timing of *A. pisum* will likely change and parthenogenetic forms of the species will be exposed to shorter day lengths than in the past. I tested whether these day length changes, possibly in combination with changes in temperature, can impose fitness constraints and thus slow phenological adaptation (Chapter III). I indeed found significant effects of day length on life-history traits of *A. pisum*, as aphids that were raised under short day length produced less offspring and reached the post-reproductive period earlier. However, the fitness constraints did not translate to the population level. I calculated population growth with a Leslie matrix, thereby taking the timing of nymph production into account, and showed that the reproductive value of the lost late-born offspring is low. This

calculation and the population level experiment show that day length does not constrain aphids strongly, at least when reproductive output is considered.

Clearly, reproductive output is not the only trait that determines inclusive fitness. It has been shown that wounded aphids increase the allocation of resources to reproduction in order to compensate for an early death (terminal investment; Barribeau et al., 2010). The challenged aphids under short days might have similarly invested more resources in earlier reproduction to compensate for the losses by short days. This reallocation would however trade off against other fitness parameters such as offspring quality, so I also raised early-and late-born aphids of the second generation on cut leaves. Unfortunately, the food quality was not sufficient for a second generation of aphids, so potential effects of day length on offspring quality could not be tested. But there are also other traits that determine aphid fitness under natural conditions, for example resistance against predation. Predation pressure and deteriorating environmental conditions induce the production of specialized winged dispersal forms (Brisson & Stern, 2006), which are more costly to produce (Braendle et al., 2006) and might thus be compromised when less resources are available. The hypothesis that day length affects wing induction remains to be tested in the future. Ultimately however, the various effects of day length on fitness need to be studied in a single experiment under field conditions.

Day length constraints of aphid predators

In contrast to *A. pisum*, the constraints of day length were more visible in the two aphid predators *Episyrphus balteatus* and *Chrysoperla carnea* (Chapter IV). While the nocturnal predator *E. balteatus* benefitted from shorter days, *C. carnea* developed slower under short days. This finding highlights the need to incorporate the whole trophic network in studies of phenology shifts. Phenology shifts for predators are generally predicted to be slower than those of herbivores (Thackeray et al., 2016), and the reported strong day length constraints of aphid predators but low constraints of their prey match this prediction. It remains however to be tested whether this is a general pattern.

I hypothesized that the day length constraints are caused by the diurnal niches of the predators. However, while *E. balteatus* is nocturnal (Holmes, 1984; Ankersmit et al., 1986), *C. carnea* does not appear to be strictly day-active (Schotzko & O'Keeffe, 1989; Joachim & Weisser, 2013; Woltz & Landis, 2014). Thus, the observed constraints are likely not only a simple action on the activity budget, but influenced by additional factors. Further studies will need to identify the diurnal rhythm in activity as well as in other physiological processes that may constrain fitness. Moreover, it would be interesting to study the circadian clock of *E. balteatus* and *C. carnea*, as I expect that the constraints are driven by strong circadian rhythms.

The trophic network illustrated in chapters III and IV is very simplified, as it covers only three species and two trophic levels. I deliberately suppressed the effects of the plant by feeding constant food sources, so the observed constraints remain to be validated in nature. Moreover, aphids rely obligately on endosymbiotic bacteria to supplement their dietary needs and to defend against parasitoids (Douglas, 2015), but these important mediators across trophic levels were not considered in my studies, so day length may exert more complex influences on trophic interactions in the field. Preliminary experiments under natural conditions have already been conducted by Bauer (2015). The study found no effect of day length on the trophic network, but the difference among the day length treatments was relatively low. Furthermore, predator composition was dominated by diurnal species and effects on aphids and on their predators might have cancelled each other out. Hence, future studies will need to concentrate on a realistic trophic network to study the effects of day length changes in nature.

Diurnality of aphids

While chapters III and IV considered day length as potential constraint of phenology shifts, the remaining chapters aimed to provide a mechanistic explanation for the constraints and focused on diurnal rhythms and the circadian clock. One might ask whether aphids require a diurnal rhythm for feeding at all. The food source is, however, not uniform over the day. The plant sap is very rich in sugar but contains only few amino acids, and how aphids thrive on this badly balanced diet has received much attention (Mittler & Koski, 1976). The C:N ratio changes due to photosynthesis over the day, and plant have diverse defence strategies whose effectiveness varies over the day/night cycle (Hevia et al., 2015). Moreover, further biotic and abiotic stressors that vary diurnally can affect feeding, e.g. temperature and humidity, or the interaction with predators or competitors. Hence I expected a diurnal rhythm, though it is difficult to predict the optimal timing of activity.

In collaboration with the Department of Neurobiology and Genetics, I showed on an artificial diet that the pea aphid has indeed a diurnal rhythm, with activity in honeydew excretion and molting peaking at day time (chapter V), which provides a likely causal mechanism of short day constraints (chapter III) by a limited time budget. The method can only give a coarse indication of the diurnal activity, as it only distinguished between day and night. Nevertheless, the study is in line with other publications on two other aphid species (Hodgson & Lane, 1981; Narayandas & Alyokhin, 2006; Taylor et al., 2012), which also concluded that aphids are day-active.

The main obstacle that precluded a higher temporal resolution was that the honeydew drops are transparent and difficult to detect under constant darkness or dim red light. I therefore improved the artificial diet, and fed various colorants to the pea aphid to make honeydew drops more visible

(chapter VI). The food colorant Brilliant Blue FCF was the most promising marker of feeding and excretion, because it stained the honeydew strongly without affecting mortality at low dosage. At the same time Katharina Beer (Department of Neurobiology and Genetics) adapted a monitor for locomotor activity of flies to aphid-specific requirements. We used these two approaches to study rhythmicity in locomotion and metabolism in a joint experiment (chapter VII). Both approaches verified that *A. pisum* is day- active, and we additionally conclude that the activity is likely bimodal. Bimodal activity patterns are not uncommon and found for example in *D. melanogaster* (Helfrich-Förster, 2000) as well as in some birds and rodents (Aschoff, 1966). Potentially, this bimodal activity pattern, constrained to early morning and late afternoon, helps aphids to avoid peak sugar concentrations. Taken together, the diurnal rhythm with activity peaking at day-time, and an associated limited time budget offers a tentative explanation of the constraints of shorter days (chapter III). Because activity does not fall fully into the photophase, it may also explain why the constraints were relatively low.

The aphid clock

So far, I have shown that shorter day length causes constraints in aphids and one aphid predator, but is beneficial for another predator. Furthermore, I linked the constraints to the diurnal rhythm of the species, hypothesizing that the time budget is either constrained or extended. While some studies showed that day length can constrain activity budgets (Zarybnicka et al., 2012), other studies found that the diurnal rhythm is a surprisingly plastic trait in nature (Fox & Bellwood, 2011). Thus, the hypothesis that diurnal rhythms cause day length constraints requires analyzing the flexibility of the activity pattern or its underlying mechanism, the circadian clock.

The circadian clock is a mechanism that runs independently of the environment and only requires occasional synchronization. Therefore circadian clocks typically persist for extended periods of time and still drive activity patterns after removing all environmental cues (Moore-Ede et al., 1982). In contrast, in our experiment the oscillations in honeydew excretion damped quickly and we detected only complex activity patterns in locomotor activity after transferal to constant conditions. Thus, we found no evidence for a strong clock in *A. pisum*, which could offer an alternative explanation why the observed day length constrains were relatively low. Comparative studies on other organisms are needed to test whether clock plasticity generally regulates the strength of day length constraints. This could either be achieved by studying the circadian clock of organisms with strong day length constraints such as *E. balteatus* or *C. carnea*, or by studying day length constraints of organisms with a known robust clock, e.g. *D. melanogaster*.

The role of the clock in plant - aphid interactions

I studied the circadian clock of aphids to evaluate the plasticity of diurnal niches, but there are also other reasons that make aphids particularly well-suited to study circadian clocks. Aphids are highly specialized phloem-sucking insects, which restricts most species to a very narrow range of host plants. Because aphids drain resources and transmit viruses, there is selection pressure on evolving resistance against aphids, and selection pressure on aphids to overcome this resistance. For these reasons aphids have been described as plant parasites (The International Aphid Genomics Consortium, 2010), and there are indeed many similarities between sap-sucking and blood-sucking insects (Guiguet et al., 2016). Circadian rhythms shape parasite-host-interactions, and coevolving circadian clocks might be one cause of speciation that is as yet overlooked (Martinez-Bakker & Helm, 2015). Hence the aphid clock is interesting on its own.

Malaria parasites suffer fitness costs if mismatched with the rhythmic mammalian host (O'Donnell et al., 2011), so they use the host rhythm to synchronize their clocks (Hotta et al., 2000). One might expect that aphids, too, synchronize with the rhythmicity of their host rather than with light. While my experiments showed that *A. pisum* has an independent rhythm (chapter V), and that light is sufficient to synchronize the aphids, there is still the possibility that changes in food quality modify the circadian phase (entrainment by feeding). I am not aware of any study that tested this hypothesis, potentially because a setup with oscillating food quality would be demanding. The improved artificial diet (chapter VI), which makes diet ingestion visible, may help in designing a protocol to measure entrainment by feeding. Overall, the circadian rhythm of aphids and its coevolution with the host plant warrant further studies.

Does the clock govern phenology?

The seasonal change of aphids has intrigued researchers since more than 100 years, but its mechanistic basis remains unknown. Before *D. melanogaster* became the universal model species, Thomas Hunt Morgan researched sex determination in gall aphids (Morgan, 1915) and in the related grape phylloxera (Morgan, 1909). He was puzzled by the apparent lack of an external signal for sex determination and concurrent life history changes (Morgan, 1909). It took another 14 years until day length was identified as the direct cue for this strategy (Marcovitch, 1923), but by then Morgan already had changed to his new model species *D. melanogaster* (Kohler, 1993). How exactly day length is measured is still under debate: The view that photoperiod measurements are based on the circadian clock (Bünning, 1936; Pittendrigh & Minis, 1964) was challenged by Lees, who saw in the aphid *Megoura viciae* evidence for hour glass clocks (Lees, 1973). In short, aphids were subjected to prolonged darkness, and then it was tested whether photoperiod sensitivity recurs

with a period of 24 hours (Nanda-Hamner and Bünsow experiments). All experiments were negative, leading to the development of the hour glass model. However, in contrast to the findings of Lees (1973), it was shown that photoperiodism is partly governed by a clock after all (Vaz Nunes & Hardie, 1993). This result can be explained by a damped clock model, in which at least two different oscillator groups contribute to photoperiodic induction, and whose influence vanishes after a few cycles (Hardie & Vaz Nunes, 2001). Yet, the damping of the clock has never been demonstrated empirically.

Our data (chapter VII) supports the damping clock model. We found complex patterns with several peaks under free-running conditions, as is predicted for a circadian system with multiple, interfering oscillators (Lewis et al., 1991). Furthermore, we showed that aphid activity is likely bimodal, and bimodal activity peaks have also been linked to multi-oscillator systems (Saunders, 2002). Lastly, we directly observed that rhythms in metabolic activity damped within 3-4 days, so our data support the damped clock model with at least two oscillators.

There are also molecular indications for a damped circadian clock. Although all core clock genes exist in *A. pisum*, they are highly diverged (Cortes et al., 2010). Especially the PER/TIM feedback loop has undergone more rapid change than other genes, implying that the clock works differently than in other organisms. Potentially functionally related, CRY2 has been duplicated, and one may tentatively conclude that these genes have acquired new function. Nevertheless, the clock appears to be functional, as mRNA transcripts of the clock genes were demonstrated to cycle under light-dark conditions, albeit with low amplitude (Cortes et al., 2010). Therefore, the molecular mechanisms can explain a quickly damping clock, which behaves like an hour glass clock in photoperiodism. Overall, the first description of the aphid clock on the behavioral level, along with novel methods to monitor clock output, will help revealing how the circadian clock and phenology are linked.

Synthesis

I have shown that novel temperature - day length correlations, which are associated with phenology shifts, can constrain fitness. Effect size and direction depend however on the species, demonstrating the need to study the whole trophic network in a natural setting. As one likely explanation of the constraints I analyzed the diurnal rhythm of aphids. I showed that aphids are day-active, which explains the day length constraints by a limited time budget. The underlying mechanism, the circadian clock, appears, however, to be extraordinarily flexible in aphids, potentially due to its role in photoperiodic time measurement. This versatility of the diurnal rhythm may explain why the constraints were relatively low in aphids; whether the time budget is more constrained in organisms with a rigid circadian clock remains to be determined. Further studies

will be needed to understand how clock and photoperiodism are linked, and how the host plant modifies the clock. The use of artificial aphid diet and food colorants can help in developing protocols to study the circadian clock in more details.

Winged form of the pea aphid.

REFERENCES

Agrawal AA. 2011. Current trends in the evolutionary ecology of plant defence. *Functional Ecology* 25:420-432.

Akey DH, and Beck SD. 1972. Nutrition of the pea aphid, *Acyrthosiphon pisum*: Requirements for trace metals, sulphur, and cholesterol. *Journal of Insect Physiology* 18:1901-1914.

Allada R, and Chung BY. 2010. Circadian organization of behavior and physiology in *Drosophila*. *Annual Review of Physiology* 72:605-624.

Anderson KJ, and Jetz W. 2005. The broad-scale ecology of energy expenditure of endotherms. *Ecology Letters* 8:310-318.

Ankersmit GW, Dijkman H, Keuning NJ, Mertens H, Sins A, and Tacoma HM. 1986. *Episyrphus balteatus* as a predator of the aphid *Sitobion avenae* on winter wheat. *Entomologia Experimentalis et Applicata* 42:271-277.

Aschoff J. 1966. Circadian activity pattern with two peaks. *Ecology* 47:657-662.

Atkinson G, and Reilly T. 1996. Circadian variation in sports performance. *Sports Medicine* 21:292-312.

Auclair JL. 1958. Honeydew excretion in the pea aphid, *Acyrthosiphon pisum* (Harr.) (Homoptera: Aphididae). *Journal of Insect Physiology* 2:330-337.

Auclair JL. 1959. Feeding and excretion by the pea aphid, *Acyrthosiphon pisum (*Harr.)(Homoptera: Aphididae), reared on different varieties of peas. *Entomologia Experimentalis et Applicata* 2:279-286.

Auclair JL. 1978. Biotypes of the pea aphid, *Acyrthosiphon piusm*, in relation to host plants and chemically defined diets. *Entomologia Experimentalis et Applicata* 24:212-216.

Auclair JL, and Cartier JJ. 1963. Pea aphid: Rearing on a chemically defined diet. *Science* 142:1068-1069.

Balvasi A, Rassoulian GR, Bandani AR, and Khalghani J. 2009. Suitability of an artificial diet for rape aphid, *Brevicoryne brassicae*, using life table parameters. *African Journal of Biotechnology* 8:4663-4666.

Barribeau SM, Sok D, and Gerardo NM. 2010. Aphid reproductive investment in response to mortality risks. *BMC Evolutionary Biology* 10.

Barton RA. 1998. Visual specialization and brain evolution in primates. *Proceedings of the Royal Society of London, Series B: Biological Sciences* 265:1933-1937.

Bass J. 2012. Circadian topology of metabolism. *Nature* 491:348-356.

Bates D, Mächler M, Bolker B, and Walker S. 2015. Fitting Linear Mixed-Effects Models Using lme4. *Journal of Statistical Software* 67:48.

Bauer S. 2015. *Auswirkungen von Desynchronisierung - durch früheres Auftreten von Blattläusen und Manipulation der Tageslichtperiode - auf Blattläuse und deren Prädatoren*. B.Sc. Thesis.

Beer K, Steffan-Dewenter I, Hartel S, and Helfrich-Förster C. 2016. A new device for monitoring individual activity rhythms of honey bees reveals critical effects of the social environment on behavior. *Journal of Comparative Physiology A* 202:555-565.

Bell JR, Alderson L, Izera D, Kruger T, Parker S, Pickup J, Shortall CR, Taylor MS, Verrier P, and Harrington R. 2015. Long-term phenological trends, species accumulation rates, aphid traits and climate: five decades of change in migrating aphids. *Journal of Animal Ecology* 84:21-34.

Bennie JJ, Duffy JP, Inger R, and Gaston KJ. 2014. Biogeography of time partitioning in mammals. *Proceedings of the National Academy of Sciences* 111:13727-13732.

Bilgi V, Fosu-Nyarko J, and Jones M. 2017. Using vital dyes to trace uptake of dsRNA by green peach aphid allows effective assessment of target gene knockdown. *International Journal of Molecular Sciences* 18:80.

Blackman RL, and Eastop VF. 2000. *Aphids on the world's crops*. Chichester, England: John Wiley & Sons.

Blix AS. 2016. Adaptations to polar life in mammals and birds. *Journal of Experimental Biology* 219:1093-1105.

Bloch G, Barnes BM, Gerkema MP, and Helm B. 2013. Animal activity around the clock with no overt circadian rhythms: patterns, mechanisms and adaptive value. *Proceedings of the Royal Society B: Biological Sciences* 280:20130019.

Boisvert JM, and Auclair JL. 1981. Influence de la riboflavine sur les besoins globaux en vitamines hydrosolubles chez le puceron du pois, *Acyrthosiphon pisum*. *canadian journal of zoology* 59:164-169.

Bouvaine S, S TB, Lin GG, Faure ML, Grebenok RJ, and Douglas AE. 2012. The physiology of sterol nutrition in the pea aphid *Acyrthosiphon pisum*. *Journal of Insect Physiology* 58:1383-1389.

Bradshaw WE, and Holzapfel CM. 2010. What season is it anyway? Circadian tracking vs. photoperiodic anticipation in insects. *Journal of Biological Rhythms* 25:155-165.

Braendle C, Davis GK, Brisson JA, and Stern DL. 2006. Wing dimorphism in aphids. *Heredity* 97:192-199.

Brisson JA, and Stern DL. 2006. The pea aphid, *Acyrthosiphon pisum*: an emerging genomic model system for ecological, developmental and evolutionary studies. *Bioessays* 28:747-755.

Bünning E. 1936. Die endogene Tagesrhythmik als Grundlage der photoperiodischen Reaktion. *Berichte der Deutschen Botanischen Gesellschaft* 54:590 - 607.

Cambier V, Hance T, and De Hoffmann E. 2001. Effects of 1,4-benzoxazin-3-one derivatives from maize on survival and fecundity of *Metopolophium dirhodum* (Walker) on artificial diet. *Journal of Chemical Ecology* 27:359-370.

Campbell A, and Mackauer M. 1977. Reproduction and population growth of the Pea Aphid (Homoptera: Aphididae) under laboratory and field conditions. *The Canadian Entomologist* 109:277-284.

CaraDonna PJ, Iler AM, and Inouye DW. 2014. Shifts in flowering phenology reshape a subalpine plant community. *Proceedings of the National Academy of Sciences* 111:4916-4921.

Carrillo L, Martinez M, Alvarez-Alfageme F, Castanera P, Smagghe G, Diaz I, and Ortego F. 2011. A barley cysteine-proteinase inhibitor reduces the performance of two aphid species in artificial diets and transgenic Arabidopsis plants. *Transgenic Research* 20:305-319.

Charmantier A, McCleery RH, Cole LR, Perrins C, Kruuk LEB, and Sheldon BC. 2008. Adaptive Phenotypic Plasticity in Response to Climate Change in a Wild Bird Population. *Science* 320:800-803.

Chaudhary R, Atamian HS, Shen Z, Briggs SP, and Kaloshian I. 2014. GroEL from the endosymbiont *Buchnera aphidicola* betrays the aphid by triggering plant defense. *Proceedings of the National Academy of Sciences* 111:8919-8924.

Chevin LM, Gallet R, Gomulkiewicz R, Holt RD, and Fellous S. 2013. Phenotypic plasticity in evolutionary rescue experiments. *Philosophical transactions of the Royal Society of London Series B, Biological sciences* 368:20120089.

Chuine I. 2010. Why does phenology drive species distribution? *Philosophical Transactions of the Royal Society of London, Series B: Biological Sciences* 365:3149-3160.

Clopton JR. 1984. Mosquito circadian and circa-bi-dian flight rhythms: a two-oscillator model. *Journal of Comparative Physiology A* 155:1-12.

Cole LC. 1954. The population consequences of life history phenomena. *The Quarterly Review of Biology* 29:103-137.

Cortes T, Ortiz-Rivas B, and Martinez-Torres D. 2010. Identification and characterization of circadian clock genes in the pea aphid *Acyrthosiphon pisum*. *Insect Molecular Biology* 19:123-139.

Dadd RH, and Mittler TE. 1966. Permanent culture of an aphid on a totally synthetic diet. *Experientia* 22:832-833.

Danks HV. 2005. How similar are daily and seasonal biological clocks? *Journal of Insect Physiology* 51:609-619.

DeWitt TJ, Sih A, and Wilson DS. 1998. Costs and limits of phenotypic plasticity. *Trends in Ecology & Evolution* 13:77-81.

Dodd AN, Salathia N, Hall A, Kévei E, Tóth R, Nagy F, Hibberd JM, Millar AJ, and Webb AAR. 2005. Plant circadian clocks increase photosynthesis, growth, survival, and competitive advantage. *Science* 309:630-633.

Douglas AE. 2015. Multiorganismal insects: diversity and function of resident microorganisms. *Annual Review of Entomology* 60:17-34.

Dunbar RIM, and Shi JB. 2013. Time as a constraint on the distribution of feral goats at high latitudes. *Oikos* 122:403-410.

Eisenbach J, and Mittler TE. 1980. An aphid circadian rhythm: Factors affecting the release of sex pheromone by oviparae of the greenbug, *Schizaphis graminum*. *Journal of Insect Physiology* 26:511-515.

Estay SA, Lima M, and Bozinovic F. 2013. The role of temperature variability on insect performance and population dynamics in a warming world. *Oikos* 123:131-140.

Evans EW, Stevenson AT, and Richards DR. 1999. Essential versus alternative foods of insect predators: benefits of a mixed diet. *Oecologia* 121:107-112.

Febvay G, Delobel B, and Rahbé Y. 1988. Influence of the amino acid balance on the improvement of an artificial diet for a biotype of *Acyrthosiphon pisum* (Homoptera: Aphididae). *canadian journal of zoology* 66:2449-2453.

Fleury F, Allemand R, Vavre F, Fouillet P, and Boulétreau M. 2000. Adaptive significance of a circadian clock: temporal segregation of activities reduces intrinsic competitive inferiority in Drosophila parasitoids. *Proceedings of the Royal Society of London, Series B: Biological Sciences* 267:1005-1010.

Fox J. 2003. Effect Displays in R for Generalised Linear Models. *2003* 8:27.

Fox RJ, and Bellwood DR. 2011. Unconstrained by the clock? Plasticity of diel activity rhythm in a tropical reef fish, *Siganus lineatus*. *Functional Ecology* 25:1096-1105.

Garner WW, and Allard HA. 1920. Effect of the relative length of day and night and other factors of the environment on growth and reproduction in plants. *Monthly Weather Review* 48:415.

Gerkema MP, Davies WIL, Foster RG, Menaker M, and Hut RA. 2013. The nocturnal bottleneck and the evolution of activity patterns in mammals. *Proceedings of the Royal Society of London, Series B: Biological Sciences* 280:20130508.

Gienapp P, Lof M, Reed TE, McNamara J, Verhulst S, and Visser ME. 2013. Predicting demographically sustainable rates of adaptation: can great tit breeding time keep pace with climate change? *Philosophical*

Transactions of the Royal Society of London, Series B: Biological Sciences 368:20120289.

Gillooly JF, Brown JH, West GB, Savage VM, and Charnov EL. 2001. Effects of size and temperature on metabolic rate. *Science* 293:2248-2251.

Golden SS. 2003. Timekeeping in bacteria: the cyanobacterial circadian clock. *Current Opinion in Microbiology* 6:535-540.

Gomez SK, Oosterhuis DM, Hendrix DL, Johnson DR, and Steinkraus DC. 2006. Diurnal pattern of aphid feeding and its effect on cotton leaf physiology. *Environmental and Experimental Botany* 55:77-86.

Goodspeed D, Chehab EW, Min-Venditti A, Braam J, and Covington MF. 2012. Arabidopsis synchronizes jasmonate-mediated defense with insect circadian behavior. *Proceedings of the National Academy of Sciences* 109:4674-4677.

Greives TJ, Kingma SA, Kranstauber B, Mortega K, Wikelski M, van Oers K, Mateman AC, Ferguson GA, Beltrami G, and Hau M. 2015. Costs of sleeping in: circadian rhythms influence cuckoldry risk in a songbird. *Functional Ecology* 29:1300-1307.

Guiguet A, Dubreuil G, Harris MO, Appel HM, Schultz JC, Pereira MH, and Giron D. 2016. Shared weapons of blood- and plant-feeding insects: Surprising commonalities for manipulating hosts. *Journal of Insect Physiology* 84:4-21.

Haeckel E. 1866. *Generelle Morphologie der Organismen. Allgemeine Grundzüge der organischen Formen- Wissenschaft, mechanisch begründet durch die von Charles Darwin reformirte Descendenz-Theorie.* . Berlin: Reimer.

Halekoh U, and Højsgaard S. 2014. A Kenward-Roger approximation and parametric bootstrap methods for tests in Linear Mixed Models - The R package pbkrtest. *Journal of Statistical Software* 59:32.

Hansen J, Sato M, Ruedy R, Lo K, Lea DW, and Medina-Elizade M. 2006. Global temperature change. *Proceedings of the National Academy of Sciences* 103:14288-14293.

Hardie J. 1989. Spectral specificity for targeted flight in the black bean aphid, *Aphis fabae*. *Journal of Insect Physiology* 35:619-626.

Hardie J, and Vaz Nunes M. 2001. Aphid photoperiodic clocks. *Journal of Insect Physiology* 47:821-832.

Hardin PE. 2011. Molecular genetic analysis of circadian timekeeping in Drosophila. *Advances in Genetics* 74:141-173.

Helfrich-Förster C. 2000. Differential control of morning and evening components in the activity rhythm of *Drosophila melanogaste*r--sex-specific differences suggest a different quality of activity. *Journal of Biological Rhythms* 15:135-154.

Helm B, Ben-Shlomo R, Sheriff MJ, Hut RA, Foster R, Barnes BM, and Dominoni D. 2013. Annual rhythms that underlie phenology: biological time-keeping meets environmental change. *Proceedings of the Royal Society of London Series B: Biological Sciences* 280:1765.

Helm B, and Visser ME. 2010. Heritable circadian period length in a wild bird population. *Proceedings of the Royal Society B: Biological Sciences* 277:3335-3342.

Hevia MA, Canessa P, Muller-Esparza H, and Larrondo LF. 2015. A circadian oscillator in the fungus *Botrytis cinerea* regulates virulence when infecting *Arabidopsis thaliana*. *Proceedings of the National Academy of Sciences* 112:8744-8749.

Hodgson CJ, and Lane IR. 1981. Some effects of photoperiod on larviposition and fresh weight-gain in *Myzus persicae*. *Physiological Entomology* 6:21-25.

Hogenhout SA, Ammar ED, Whitfield AE, and Redinbaugh MG. 2008. Insect vector interactions with persistently transmitted viruses. *Annual Review of Phytopathology* 46:327-359.

Holmes PR. 1984. A field study of the predators of the grain aphid, *Sitobion avenae* (F.) (Hemiptera: Aphididae), in winter wheat in Britain. *Bulletin of Entomological Research* 74:623-631.

Hondelmann P, and Poehling H-M. 2007. Diapause and overwintering of the hoverfly *Episyrphus balteatus*. *Entomologia Experimentalis et Applicata* 124:189-200.

Hooven LA, Sherman KA, Butcher S, and Giebultowicz JM. 2009. Does the clock make the poison? Circadian variation in response to pesticides. *Plos One* 4:e6469.

Hotta CT, Gazarini ML, Beraldo FH, Varotti FP, Lopes C, Markus RP, Pozzan T, and Garcia CRS. 2000. Calcium-dependent modulation by melatonin of the circadian rhythm in malarial parasites. *Nature Cell Biology* 2:466-468.

Hsu PY, and Harmer SL. 2014. Wheels within wheels: the plant circadian system. *Trends in Plant Science* 19:240-249.

Huang X-L, and Qiao G-X. 2014. Aphids as models for ecological and evolutionary studies. *Insect Science* 21:247-250.

Ikeno T, Tanaka S, Numata H, and Goto S. 2010. Photoperiodic diapause under the control of circadian clock genes in an insect. *BMC Biology* 8:116.

IPCC. 2000. *Special Report on Emissions Scenarios*. Cambridge, U.K.: Cambridge University Press.

IPCC. 2007. *Climate Change 2007: The Physical Science Basis. Contribution of Working Group I to the Fourth Assessment Report of the Intergovernmental Panel on Climate Change*. Cambridge: Cambridge University Press.

IPCC. 2013. *Climate Change 2013: The physical science basis. Working Group I contribution to the fifth assessment report of the Intergovernmental Panel on Climate Change*. New York: Cambridge University Press.

IPCC. 2014. *Climate Change 2014: Synthesis Report. Contribution of Working Groups I, II and III to the Fifth Assessment Report of the Intergovernmental Panel on Climate Change*. Geneva, Switzerland: IPCC.

Jaouannet M, Rodriguez PA, Thorpe P, Lenoir CJG, MacLeod R, Escudero-Martinez C, and Bos JIB. 2014. Plant immunity in plant-aphid interactions. *Frontiers in Plant Science* 5:663.

Jing X, White TA, Luan J, Jiao C, Fei Z, and Douglas AE. 2016. Evolutionary conservation of candidate osmoregulation genes in plant phloem sap-feeding insects. *Insect Molecular Biology* 25:251–258.

Joachim C, and Weisser W. 2013. Real-Time monitoring of (E)-β-Farnesene emission in colonies of the pea aphid, *Acyrthosiphon pisum*, under lacewing and ladybird Predation. *Journal of Chemical Ecology* 39:1254-1262.

Joschinski J, Beer K, Helfrich-Förster C, and Krauss J. 2016. Pea aphids (Hemiptera: Aphididae) have diurnal rhythms when raised independently of a host plant. *Journal of Insect Science* 16:1-5.

Joschinski J, Hovestadt T, and Krauss J. 2015. Coping with shorter days: do phenology shifts constrain aphid fitness? *PeerJ* 3:e1103.

Kaakeh W, and Dutcher JD. 1993. Rates of increase and probing behavior of *Acyrthosiphon pisum*(Homoptera: Aphididae) on preferred and nonpreferred host cover crops. *Environmental Entomology* 22:1016-1021.

Kauranen H, Menegazzi P, Costa R, Helfrich-Förster C, Kankainen A, and Hoikkala A. 2012. Flies in the north: locomotor behavior and clock neuron organization of *Drosophila montana*. *Journal of Biological Rhythms* 27:377-387.

Kenten J. 1955. The Effect of photoperiod and temperature on reproduction in *Acyrthosiphon pisum* (Harris) and on the forms produced. *Bulletin of Entomological Research* 46:599-624.

Kilian L, and Nielson MW. 1971. Differential Effects of Temperature on the Biological Activity of Four Biotypes of the Pea Aphid. *Journal of Economic Entomology* 64:153-155.

Kislow CJ, and Edwards LJ. 1972. Repellent odour in aphids. *Nature* 235:108-109.

Kloth KJ, ten Broeke CJ, Thoen MP, Hanhart-van den Brink M, Wiegers GL, Krips OE, Noldus LP, Dicke M, and Jongsma MA. 2015. High-throughput phenotyping of plant resistance to aphids by automated video tracking. *Plant Methods* 11:4.

Kohler RE. 1993. Drosophila: A life in the laboratory. *Journal of the History of Biology* 26:281-310.

Kostal V. 2011. Insect photoperiodic calendar and circadian clock: Independence, cooperation, or unity? *Journal of Insect Physiology* 57:538-556.

Krieger DL. 1971. Rearing several aphid species on synthetic diet. *Annals of the Entomological Society of America* 64:1176-1177.

Kronfeld-Schor N, Bloch G, and Schwartz WJ. 2013. Animal clocks: when science meets nature. *Proceedings of the Royal Society B: Biological Sciences* 280:1765.

Kronfeld-Schor N, and Dayan T. 2003. Partitioning of time as an ecological resource. *Annual Review of Ecology Evolution and Systematics* 34:153-181.

Kunkel H, and Hertel R. 1976. Kotabgabe von holidisch ernährten *Myzus persicae* - Larven (Aphidina, Homoptera) bei unterschiedlichen Experimentalbedingungen. *Entomologia Experimentalis et Applicata* 19:82-95.

Lees AD. 1959. The role of photoperiod and temperature in the determination of parthenogenetic and sexual forms in the aphid *Megoura viciae* Buckton—I. *Journal of Insect Physiology* 3:92-117.

Lees AD. 1973. Photoperiodic time measurement in the aphid *Megoura viciae*. *Journal of Insect Physiology* 19:2279-2316.

Lees AD. 1986. Some effects of temperature on the hour glass photoperiod timer in the aphid *Megoura viciae*. *Journal of Insect Physiology* 32:79-89.

Leslie PH. 1945. On the use of matrices in certain population mathematics. *Biometrika* 33:183-212.

Lewis RD, Bullivant AG, and King VM. 1991. A dual pacemaker model for the circadian system of the insect *Hemideina thoracica*. *Journal of Interdisciplinary Cycle Research* 22:293-309.

Losey JE, and Denno RF. 1998. The escape response of pea aphids to foliar-foraging predators: factors affecting dropping behaviour. *Ecological Entomology* 23:53-61.

Lu WQ, Meng QJ, Tyler NJC, Stokkan KA, and Loudon ASI. 2010. A circadian clock is not required in an arctic mammal. *Current Biology* 20:533-537.

Mahendiran M. 2016. Coexistence of three sympatric cormorants (*Phalacrocorax spp.*); partitioning of time as an ecological resource. *Royal Society Open Science* 3:8.

Marcovitch S. 1923. Plant lice and light exposure. *Science* 58:537-538.

Marques MD, and Waterhouse J. 2004. Rhythms and Ecology — do chronobiologists still remember nature? *Biological Rhythm Research* 35:1-2.

Martinez-Bakker M, and Helm B. 2015. The influence of biological rhythms on host–parasite interactions. *Trends in Ecology & Evolution* 30:314-326.

McCarthy K, Kjærsgaard A, Bahrndorff S, Schou TM, Manenti T, Loeschcke V, and Pertoldi C. 2015. The Effect of Social Isolation on Locomotor Activity in the Houseflies (*Musca Domestica*). *Journal of Insect Behavior* 28:288-296.

McLean DL, and Kinsey MG. 1964. A technique for electronically recording aphid feeding and salivation. *Nature* 202:1358-1359.

Meister B, Krauss J, Härri SA, Schneider MV, and Müller CB. 2006. Fungal endosymbionts affect aphid population size by reduction of adult life span and fecundity. *Basic and Applied Ecology* 7:244-252.

Menegazzi P, Yoshii T, and Helfrich-Förster C. 2012. Laboratory versus nature: the two sides of the Drosophila circadian clock. *Journal of Biological Rhythms* 27:433-442.

Mittler TE. 1976. Ascorbic acid and other chelating agents in the trace-mineral nutrition of the aphid *Myzus persicae* on artificial diets. *Entomologia Experimentalis et Applicata* 20:81-98.

Mittler TE, and Dadd RH. 1962. Artificial feeding and rearing of the aphid, *Myzus persicae* (Sulzer), on a completely defined synthetic diet. *Nature* 195:404-404.

Mittler TE, and Dadd RH. 1963. Studies on the artificial feeding of the aphid *Myzus persicae* (Sulzer)—I. Relative uptake of water and sucrose solutions. *Journal of Insect Physiology* 9:623-645.

Mittler TE, and Koski P. 1976. Development of meridic and oligidic diets for rearing the aphid *Myzus persicae*. *Journal of Insect Physiology* 22:1135-1141.

Mohawk JA, Green CB, and Takahashi JS. 2012. Central and peripheral circadian clocks in mammals. *Annual Review of Neuroscience* 35:445-462.

Moore-Ede MC, Sulzman FM, and Fuller CA. 1982. *The clocks that time us*. Cambridge, MA: Harvard University Press.

Morgan TH. 1909. Sex determination and parthenogenesis in phylloxerans and aphids. *Science* 29:234-237.

Morgan TH. 1915. The predetermination of sex in phylloxerans and aphids. *Journal of Experimental Zoology* 19:285-321.

Mrosovsky N, and Hattar S. 2005. Diurnal mice (*Mus musculus*) and other examples of temporal niche switching. *Journal of Comparative Physiology A* 191:1011-1024.

Nakabachi A, and Ishikawa H. 1999. Provision of riboflavin to the host aphid, A*cyrthosiphon pisum*, by endosymbiotic bacteria, *Buchnera*. *Journal of Insect Physiology* 45:1-6.

Narayandas G, and Alyokhin A. 2006. Diurnal patterns in host finding by potato aphids, *Macrosiphum euphorbiae* (Homoptera: Aphididae). *Journal of Insect Behavior* 19:347-356.

O'Donnell AJ, Schneider P, McWatters HG, and Reece SE. 2011. Fitness costs of disrupting circadian rhythms in malaria parasites. *Proceedings of the Royal Society B: Biological Sciences* 278:2429-2436.

Paguia P, Pathak MD, and Heinrichs EA. 1980. Honeydew excretion measurement techniques for determining differential feeding activity of biotypes of *Nilaparvata lugens* on rice varieties. *Journal of Economic Entomology* 73:35-40.

Parmesan C, and Yohe G. 2003. A globally coherent fingerprint of climate change impacts across natural systems. *Nature* 421:37-42.

Paula DP, and Andow DA. 2016. Differential Cry toxin detection and effect on *Brevicoryne brassicae* and *Myzus persicae* feeding on artificial diet. *Entomologia Experimentalis et Applicata* 159:54-60.

Payne NL, van der Meulen DE, Gannon R, Semmens JM, Suthers IM, Gray CA, and Taylor MD. 2013. Rain reverses diel activity rhythms in an estuarine teleost. *Proceedings of the Royal Society B: Biological Sciences* 280:7.

Peschel N, and Helfrich-Förster C. 2011. Setting the clock - by nature: Circadian rhythm in the fruitfly *Drosophila melanogaster*. *FEBS Letters* 585:1435-1442.

Petrusek A, Tollrian R, Schwenk K, Haas A, and Laforsch C. 2009. A “crown of thorns” is an inducible defense that protects *Daphnia* against an ancient predator. *Proceedings of the National Academy of Sciences* 106:2248-2252.

Pirone TP, and Harris KF. 1977. Nonpersistent transmission of plant viruses by aphids. *Annual Review of Phytopathology* 15:55-73.

Pittendrigh CS, and Minis DH. 1964. The entrainment of circadian oscillations by light and their role as photoperiodic clocks. *American Naturalist* 98:261-294.

Pittendrigh CS, and Minis DH. 1972. Circadian systems: longevity as a function of circadian resonance in *Drosophila melanogaster*. *Proceedings of the National Academy of Sciences of the United States of America* 69:1537-1539.

Pivarciova L, Vaneckova H, Provaznik J, Wu BC-h, Pivarci M, Peckova O, Bazalova O, Cada S, Kment P, Kotwica-Rolinska J et al. . 2016. Unexpected geographic variability of the free running period in the linden bug *Pyrrhocoris apterus*. *Journal of Biological Rhythms* 31:568-576.

Pizzatto L, Child T, and Shine R. 2008. Why be diurnal? Shifts in activity time enable young cane toads to evade cannibalistic conspecifics. *Behavioral Ecology* 19:990-997.

Poesel A, Kunc HP, Foerster K, Johnsen A, and Kempenaers B. 2006. Early birds are sexy: male age, dawn song and extrapair paternity in blue tits, *Cyanistes* (formerly *Parus*) *caeruleus*. *Animal Behaviour* 72:531-538.

Powell G, Tosh CR, and Hardie J. 2006. Host plant selection by aphids: behavioral, evolutionary, and applied perspectives. *Annual Review of Entomology* 51:309-330.

Qadeer A, Raza M, Nazir A, Javed A, Shahnaz N, Hayder A, and Nazia S. 2012. Effect of different Photo periods on the biological parameters of *Chrysoperla carnea* under laboratory conditions. *Journal of Basic & Applied Sciences* 8:638-640.

R Core Team. 2012. R: A language and environment for statistical computing. Vienna, Austria: R Foundation for Statistical Computing.

R Core Team. 2014. R: A language and environment for statistical computing. Vienna, Austria: R Foundation for Statistical Computing.

Rabus M, and Laforsch C. 2011. Growing large and bulky in the presence of the enemy: *Daphnia magna* gradually switches the mode of inducible morphological defences. *Functional Ecology* 25:1137-1143.

Räisänen J, and Eklund J. 2012. 21st Century changes in snow climate in Northern Europe: a high-resolution view from ENSEMBLES regional climate models. *Climate Dynamics* 38:2575-2591.

Ramsey JS, Wilson AC, de Vos M, Sun Q, Tamborindeguy C, Winfield A, Malloch G, Smith DM, Fenton B, Gray SM et al. . 2007. Genomic resources for *Myzus persicae:* EST sequencing, SNP identification, and microarray design. *BMC Genomics* 8:1-17.

Rao Q, Rollat-Farnier P-A, Zhu D-T, Santos-Garcia D, Silva FJ, Moya A, Latorre A, Klein CC, Vavre F, Sagot M-F et al. . 2015. Genome reduction and potential metabolic complementation of the dual endosymbionts in the whitefly *Bemisia tabaci*. *BMC Genomics* 16:226.

Refinetti R, Lissen GC, and Halberg F. 2007. Procedures for numerical analysis of circadian rhythms. *Biological Rhythm Research* 38:275-325.

Reppert SM, and Weaver DR. 2002. Coordination of circadian timing in mammals. *Nature* 418:935-941.

Rispe C, Simon JC, and Pierre JS. 1996. Fitness comparison between clones differing in their ability to produce sexuals in the aphid *Rhopalosiphum padi*. *Entomologia Experimentalis et Applicata* 80:469-474.

Roenneberg T, Daan S, and Merrow M. 2003a. The art of entrainment. *Journal of Biological Rhythms* 18:183-194.

Roenneberg T, Wirz-Justice A, and Merrow M. 2003b. Life between clocks: daily temporal patterns of human chronotypes. *Journal of Biological Rhythms* 18:80-90.

Root TL, Price JT, Hall KR, Schneider SH, Rosenzweig C, and Pounds JA. 2003. Fingerprints of global warming on wild animals and plants. *Nature* 421:57-60.

Rosenzweig C, Casassa G, Karoly DJ, Imeson A, Liu C, Menzel A, Rawlins S, Root TL, Seguin B, and Tryjanowski P. 2007. Assessment of observed changes and responses in natural and managed systems. In: M.L. Parry, O.F. Canziani, J.P. Palutikof, P.J. van der Linden, and Hanson CE, eds. *Climate Change 2007: Impacts, Adaptation and Vulnerability Contribution of Working Group II to the Fourth Assessment Report of the Intergovernmental Panel on Climate Change*. Cambridge, UK: Cambridge University Press, 79-131.

Ruf T. 1999. The Lomb-Scargle Periodogram in biological rhythm research: analysis of incomplete and unequally spaced time-sesries. *Biological Rhythm Research* 30:178-201.

Sadeghi A, Van Damme EJM, Michiels K, Kabera A, and Smagghe G. 2009. Acute and chronic insecticidal activity of a new mannose-binding lectin from *Allium porrum* against A*cyrthosiphon pisum* via an artificial diet. *Canadian Entomologist* 141:95-101.

Santos RG, Pinheiro HT, Martins AS, Riul P, Bruno SC, Janzen FJ, and Ioannou CC. 2016. The anti-predator role of within-nest emergence synchrony in sea turtle hatchlings. *Proceedings of the Royal Society B: Biological Sciences* 283:1834.

Sasaki T, Hayashi H, and Ishikawa H. 1991. Growth and reproduction of the symbiotic and aposymbiotic pea aphids, *Acyrthosiphon pisum* maintained on artificial diets. *Journal of Insect Physiology* 37:749-756.

Saunders DS. 2002. *Insect Clocks, Third Edition*. Amsterdam: Elsevier Science.

Saunders DS. 2005. Erwin Bünning and Tony Lees, two giants of chronobiology, and the problem of time measurement in insect photoperiodism. *Journal of Insect Physiology* 51:599-608.

Saunders DS. 2013. Insect photoperiodism: Measuring the night. *Journal of Insect Physiology* 59:1-10.

Saunders DS. 2014. Insect photoperiodism: effects of temperature on the induction of insect diapause and diverse roles for the circadian system in the photoperiodic response. *Entomological Science* 17:25-40.

Schaefer CW. 1938. Physiological conditions which produce wing development in the pea aphid. *Journal of Agricultural Research* 57.

Schmid B, Helfrich-Forster C, and Yoshii T. 2011. A new ImageJ plug-in "ActogramJ" for chronobiological analyses. *Journal of Biological Rhythms* 26:464-467.

Schotzko DJ, and O'Keeffe LE. 1989. Comparison of Sweep Net, D-Vac, and Absolute Sampling, and diel variation of sweep net sampling estimates in lentils for pea aphid (Homoptera: Aphididae), nabids (Hemiptera:

Nabidae), lady beetles (Coleoptera: Coccinellidae), and lacewings (Neuroptera: Chrysopidae). *Journal of Economic Entomology* 82:491-506.

Schultz TF, and Kay SA. 2003. Circadian clocks in daily and seasonal control of development. *Science* 301:326-328.

Shibao H, Kutsukake M, Lee JM, and Fukatsu T. 2002. Maintenance of soldier-producing aphids on an artificial diet. *Journal of Insect Physiology* 48:495-505.

Simon J-C, Rispe C, and Sunnucks P. 2002. Ecology and evolution of sex in aphids. *Trends in Ecology & Evolution* 17:34-39.

Stich H-B, and Lampert W. 1981. Predator evasion as an explanation of diurnal vertical migration by zooplankton. *Nature* 293:396-398.

Styrsky JD, and Eubanks MD. 2007. Ecological consequences of interactions between ants and honeydew-producing insects. *Proceedings of the Royal Society B: Biological Sciences* 274:151-164.

Tauber MJ, and Tauber CA. 1969. Diapause in *Chrysopa carnea* (Neuroptera: Chrysopidae): I. Effect of photoperiod on reproductively active adults. *The Canadian Entomologist* 101:364-370.

Taylor SH, Parker WE, and Douglas AE. 2012. Patterns in aphid honeydew production parallel diurnal shifts in phloem sap composition. *Entomologia Experimentalis et Applicata* 142:121-129.

Tessmar-Raible K, Raible F, and Arboleda E. 2011. Another place, another timer: Marine species and the rhythms of life. *Bioessays* 33:165-172.

Thackeray SJ, Henrys PA, Hemming D, Bell JR, Botham MS, Burthe S, Helaouet P, Johns DG, Jones ID, Leech DI et al. . 2016. Phenological sensitivity to climate across taxa and trophic levels. *Nature* 535:241-245.

The International Aphid Genomics Consortium. 2010. Genome sequence of the Pea aphid *Acyrthosiphon pisum*. *PLoS Biology* 8:e1000313.

Thieme T, and Dixon AFG. 1996. Mate recognition in the *Aphis fabae* complex: daily rhythm of release and specificity of sex pheromones. *Entomologia Experimentalis et Applicata* 79:85-89.

van Emden H. 2009. Artificial diet for aphids - Thirty years' experience. *Redia* 92:163-167.

van Emden HF, Dingley J, Dewhirst SY, Pickett JA, Woodcock CM, and Wadhams LJ. 2014. The effect of artificial diet on the production of alarm pheromone by *Myzus persicae*. *Physiological Entomology* 39:285-291.

van Emden HF, and Harrington R. 2007. *Aphids as Crop Pests*. Wallingford, United Kingdom: Cabi.

Vaz Nunes M, and Hardie J. 1987. An instantly damping oscillator model for photoperiodic time measurement in the aphid *Aphis fabae*. *Journal of Insect Physiology* 33:831-841.

Vaz Nunes M, and Hardie J. 1993. Circadian rhythmicity is involved in photoperiodic time measurement in the aphid *Megoura viciae*. *Experientia* 49:711-713.

Vaze KM, and Helfrich-Förster C. 2016. *Drosophila ezoana* uses an hour-glass or highly damped circadian clock for measuring night length and inducing diapause. *Physiological Entomology* 41:378-389.

Vaze KM, and Sharma VK. 2013. On the adaptive significance of circadian clocks for their owners. *Chronobiology International* 30:413-433.

Vedder O, Bouwhuis S, and Sheldon BC. 2013. Quantitative assessment of the importance of phenotypic plasticity in adaptation to climate change in wild bird populations. *PLoS Biology* 11.

Visser ME, Both C, and Lambrechts MM. 2004. Global climate change leads to mistimed avian reproduction. *Advances in Ecological Research*. Oxford, United Kingdom: Academic Press, 89-110.

Visser ME, Caro SP, Oers Kv, Schaper SV, and Helm B. 2010. Phenology, seasonal timing and circannual rhythms: towards a unified framework. *Philosophical Transactions of the Royal Society of London, Series B: Biological Sciences* 365:3113-3127.

Visser ME, and Holleman LJM. 2001. Warmer springs disrupt the synchrony of oak and winter moth phenology. *Proceedings of the Royal Society of London Series B: Biological Sciences* 268:289-294.

Visser ME, Noordwijk AJv, Tinbergen JM, and Lessells CM. 1998. Warmer springs lead to mistimed reproduction in great tits (*Parus major*). *Proceedings of the Royal Society B-Biological Sciences* 265:1867-1870.

Vitasse Y, Bresson CC, Kremer A, Michalet R, and Delzon S. 2010. Quantifying phenological plasticity to temperature in two temperate tree species. *Functional Ecology* 24:1211-1218.

Vitt LJ, and Zug GR. 2012. *Herpetology: An Introductory Biology of Amphibians and Reptiles*. Oxford, United Kingdom: Academic Press.

Whitehead K, Pan M, Masumura K, Bonneau R, and Baliga NS. 2009. Diurnally Entrained Anticipatory Behavior in Archaea. *Plos One* 4.

Wier AM, Nyholm SV, Mandel MJ, Massengo-Tiassé RP, Schaefer AL, Koroleva I, Splinter-BonDurant S, Brown B, Manzella L, Snir E et al. . 2010. Transcriptional patterns in both host and bacterium underlie a daily rhythm of anatomical and metabolic change in a beneficial symbiosis. *Proceedings of the National Academy of Sciences* 107:2259-2264.

Wille BD, and Hartman GL. 2008. Evaluation of artificial diets for rearing *Aphis glycines* (Hemiptera : Aphididae). *Journal of Economic Entomology* 101:1228-1232.

Willett DS, George J, Willett NS, Stelinski LL, and Lapointe SL. 2016. Machine Learning for Characterization of Insect Vector Feeding. *PLoS Computational Biology* 12:e1005158.

Woltz JM, and Landis DA. 2014. Comparison of sampling methods of *Aphis glycines* predators across the diel cycle. *Journal of Applied Entomology* 138:475-484.

Wood BW, Tedders WL, and Reilly CC. 1988. Sooty mold fungus on pecan foliage suppresses light penetration and net photosynthesis. *HortScience* 23:851-853.

Ximenez-Embun MG, Zaviezo T, and Grez A. 2014. Seasonal, spatial and diel partitioning of *Acyrthosiphon pisum* (Hemiptera: Aphididae) predators and predation in alfalfa fields. *Biological Control* 69:1-7.

Zarybnicka M, Korpimaki E, and Griesser M. 2012. Dark or short nights: differential latitudinal constraints in nestling provisioning patterns of a nocturnally hunting bird species. *Plos One* 7:9.

Zhu H, Sauman I, Yuan Q, Casselman A, Emery-Le M, Emery P, and Reppert SM. 2008. Cryptochromes cefine a novel circadian clock mechanism in monarch butterflies that may underlie sun compass navigation. *PLoS Biology* 6:e4.

Zimova M, Mills LS, and Nowak JJ. 2016. High fitness costs of climate change-induced camouflage mismatch. *Ecology Letters* 19:299-307.

Züst T, and Agrawal AA. 2016. Mechanisms and evolution of plant resistance to aphids. *Nature Plants* 2:15206.

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Oral presentations at international conferences

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Joschinski J, Krauss, J. (2015). Climate change and phenology shifts: does a changing time budget constrain aphids? British Ecological Society annual meeting 2015, Edinburgh, UK.

Joschinski J, Krauss, J. (2015). Day length constraints and diurnal activity of the pea aphid. Joint meeting of the French Aphid Research Network and the Aphid Special Interest Group of the Royal Entomological Society, Paris, France.

Joschinski J, Hovestadt T, Krauss, J. (2015). Do phenology shifts cause day length constraints in aphids? 45th annual Meeting of the Ecological Society of Germany, Austria and Switzerland. Göttingen, Germany.

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PUBLICATIONS

Published manuscripts

Joschinski J, Beer K, Helfrich-Förster C, Krauss J. (2016) Pea aphids (Hemiptera: Aphididae) have diurnal rhythms when raised independently of a host plant. *Journal of Insect Science*, 16: 1-5. DOI: 10.1093/jisesa/iew013.

Joschinski J, Hovestadt T, Krauss J. (2015) Coping with shorter days: do phenology shifts constrain aphid fitness? *PeerJ*, 3: e1103. DOI: 10.7717/peerj.1103.

Joschinski J, van Kleunen M, Stift M. (2015) Costs associated with the evolution of selfing in North American populations of *Arabidopsis lyrata*? *Evolutionary Ecology,* 29: 749-764. DOI: 10.1007/s10682-015-9786-3.

Joschinski J. (2016) Benefits and costs of aphid phenological bet-hedging strategies. . *Research Ideas and Outcomes,* 2: e9580. DOI: 10.3897/rio.2.e9580. **Published idea, not peer-reviewed**

Unpublished manuscripts

Joschinski J, Krauss J. Food colouring as new possibility to study diet ingestion and honeydew excretion by aphids. **Under review.**

Joschinski J, Kiess T, Krauss J. Day length constrains the time budget of aphid predators**. Under review.**

Beer K[1]**, Joschinski J[1],** Helfrich-Förster C[2], Krauss J[2]. A damping circadian clock drives weak oscillations in metabolism and locomotor activity of aphids (*Acyrthosiphon pisum*). **In preparation**.

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STATEMENT OF INDIVIDUAL AUTHOR CONTRIBUTIONS TO FIGURES/TABLES/CHAPTERS

Publication : **Joschinski J,** Hovestadt, T., & Krauss, J. (2015) Coping with shorter days: do phenology shifts constrain aphid fitness? *PeerJ*, **3**, e1103.					
Figure	**Author Initials,** Responsibility decreasing from left to right				
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Data Collection	KB	JJ	CHF		
Data Analysis and Interpretation	JJ	KB	JK		
Manuscript Writing Writing of Introduction Writing of Materials & Methods Writing of Discussion Writing of First Draft	 JJ JJ KB KB	 KB KB JJ JJ	 JK JK CHF CHF		

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Whether fully aware of it or not, we human beings are immersed in a world of insects.

T.Eisner and **Edward O. Wilson**, The Insects: Readings from Scientific American

Chaitophorus salijaponicus (Essig & Kuwana), collected in Würzburg. Despite its broad distribution (being recorded in East Asia, Italy and Greece), a search with google images reveals no record of the species.