Effects of different light regimes on the resistance of *Nicotiana attenuata* against *Manduca sexta*

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ii. Abbreviations

*A. thaliana*  *Arabidopsis thaliana*

CGA  chlorogenic acid

CP  N-caffeoylputrescine

DD  continuous dark conditions

ESI  electrospray ionization source

HGL-DTGs  17-hydroxygeranyllinalool-diterpene glycosides

JA  jasmonic acid or jasmonates

JA-Ile  jasmonic acid – isoleucine conjugate

LL  continuous light conditions

*M. sexta*  *Manduca sexta*

MeOH  methanol

MS  mass spectrometry

*N. attenuata*  *Nicotiana attenuata*

PAR  photosynthetically active radiation

PCA  principal component analysis

R:FR  red to far-red ratio

SAS  shade avoidance syndrome

*T. ni*  *Trichoplusia ni*

UPLC/HPLC  ultra-high pressure liquid chromatography/high pressure liquid chromatography

WT  wild type
1. Introduction

1.1. Circadian clocks – generalities and specifics

Through the Earth’s rotation, organisms experience the change of day to night in a ca. 24 h cycle, and most have developed an internal circadian clock in order to synchronize their activities with predictable diurnal events (Young and Kay 2001, Bell-Pedersen et al. 2005, Mackey 2007). Circadian rhythms are found in all three domains of life, namely Archaea, Bacteria and Eucarya (Koukkari and Sothern 2006, Mackey 2007, Whitehead et al. 2009) which display profound differences in the make-up of their circadian clocks (Mackey 2007). However, recently a potential circadian pacemaker conserved across all domains was identified: a circadian rhythm in the redox status of peroxiredoxin (Edgar et al. 2012), associated with the cellular response to reactive oxygen species, and might represent an evolutionary older circadian clock (Edgar et al. 2012). The eukaryotic clock has been closely investigated in animals (mouse, humans, fruit fly), fungi (Neurospora crassa) and plants (Arabidopsis thaliana)(Mackey 2007, Nagel and Kay 2012) and a shared general feature of the eukaryotic clockwork oscillator is an auto-regulatory feedback loop, in which the components regulate each other, primarily via transcriptional regulation, but also, as indicated by recent findings, via post-transcriptional and post-translational regulation and chromatin remodeling (Nagel and Kay 2012).

Biological rhythms can be distinguished by a set of characteristics (Fig. 1A). Not all biological rhythms are circadian; circadian rhythms are distinguished by their period length which, under diurnal conditions, is entrained (= synchronized) to the 24h day length of the natural environment, and maintains a free-running rhythm of approximately 24h (defined as: 20-28 h) under constant conditions (Fig. 1B) (Koukkari and Sothern 2006, Harmer 2009). Diurnal rhythms, on the other hand, cease upon transfer to constant conditions (Koukkari and Sothern 2006) Thus, circadian rhythms display entrainability to environmental cues and a self-sustained oscillation of ~24h. Furthermore, circadian rhythms are characterized by temperature-compensation and maintain a similar period length over a range of temperatures (McClung 2006).
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A) Oscillations can be characterized by their amplitude and period length. The amplitude is half the distance between peak and trough. The period length is the time-interval usually measured between two subsequent peaks or troughs of an oscillation. The phase is the time of day for any given event or position within the oscillation. Definitions are from McClung (2006) and Koukkari and Sothern (2006). The figure was re-drawn after figure 1 in McClung (2006).

B) Phase is often defined in zeitgeber time (ZT; a time cue is a zeitgeber) under diurnal conditions, and as circadian time (CT; relative to subjective dawn) under constant conditions. The phase of actual or expected dawn is defined as ZT0 or CT0, respectively. A circadian rhythm can be entrained to 24 h period length under a diurnal entrainment regime, but, upon release into constant conditions, will persist briefly in its former entrained period length, but might soon show a slight shortening or lengthening in its period length. This period length demonstrated under constant conditions is the free-running, endogenous, circadian-clock generated period of the circadian rhythm. Diurnal conditions are indicated by white and dark grey bars, constant light conditions are indicated by white (continued from page 3) and light grey bars; light grey bars indicate

Figure 1: Characteristics of circadian rhythms. A) Oscillations can be characterized by their amplitude and period length. The amplitude is half the distance between peak and trough. The period length is the time-interval usually measured between two subsequent peaks or troughs of an oscillation. The phase is the time of day for any given event or position within the oscillation. Definitions are from McClung (2006) and Koukkari and Sothern (2006). The figure was re-drawn after figure 1 in McClung (2006). B) Phase is often defined in zeitgeber time (ZT; a time cue is a zeitgeber) under diurnal conditions, and as circadian time (CT; relative to subjective dawn) under constant conditions. The phase of actual or expected dawn is defined as ZT0 or CT0, respectively. A circadian rhythm can be entrained to 24 h period length under a diurnal entrainment regime, but, upon release into constant conditions, will persist briefly in its former entrained period length, but might soon show a slight shortening or lengthening in its period length. This period length demonstrated under constant conditions is the free-running, endogenous, circadian-clock generated period of the circadian rhythm. Diurnal conditions are indicated by white and dark grey bars, constant light conditions are indicated by white (continued from page 3) and light grey bars; light grey bars indicate
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(continued from page 8) subjective night. Definitions are from McClung, (2001, 2006) and Harmer (2009). The figure was redrawn after figure 1 in Harmer (2009).

1.2. The circadian clock of plants

Light is the most important entrainment cue for plant clocks, as for most or all other organisms. Light input to the circadian plant clock can be red or blue wavelengths; perception of the former is via phytochromes (PHYA-E) and perception of the latter is via cryptochromes (CRY1 and CRY2) (Franklin 2008), but the mechanism of action of the phytochromes and cryptochromes on the clock is not fully understood (McWatters and Devlin 2011). Similarly, while the plant clock can also entrain to temperature cycles, the mechanism of perception is unknown to date (McWatters and Devlin 2011). Recent studies have implied that the clock might also be entrainable via sugar availability, by an unknown mechanism (Dalchau et al. 2011, Haydon et al. 2013).

A simplified representation of the plant circadian clock is that shown in Fig. 2 (based on the current theoretical model published by Pokhilko et al. (2012)). Outputs of the plant circadian clock are manifold, including, among others, growth processes, e.g. in seedlings of *A. thaliana*, timing of hypocotyl elongation to a specific time of day (Nozue et al. 2007), regulation of phytohormones (e.g. auxin, (Covington and Harmer 2007); jasmonates and salicylates (Goodspeed et al. 2012) and regulation of starch metabolism (Graf et al. 2010). Furthermore, certain photoperiodic responses are under the direct control of the clock, e.g. earlier flowering of *A. thaliana* under long-day conditions (Suárez-López et al. 2001). (For clock outputs in respect to diurnal abiotic stressors and biotic interactions, see section 1.3. below.) Furthermore, there is strong transcriptional control of gene expression in *A. thaliana* attributed to the circadian clock (Covington et al. 2008).
Figure 2: A conceptual model of the plant circadian system, simplified as a linear signaling pathway. The plant circadian clock is represented as comprising three discrete components: A) Input mechanisms that change the phase of the clock in response to environmental stimuli, B) a central oscillator and C) a variety of rhythmic outputs. Input to the circadian plant clock can be via light (Franklin 2008), via temperature cycles (McWatters and Devlin 2011) and probably even via sugar availability (Dalchau et al. 2011, Haydon et al. 2013). In the current theoretical model of the central oscillator of the plant circadian clock (Pokhilko et al. 2012), the simplest representation of the plant circadian clock (Figure 2B) and its most important components is a repressilator, a three-inhibitor ring oscillator. The three components comprise CCA1/LHY and PRRs 9/7/5, as morning loop components, and the auto-regulatory evening complex, a transcriptionally active protein complex comprising ELF3/ELF4/LUX that closes the evening loop by negatively regulating the transcription of its own elements. Each of the three components is negatively regulated by the respective later-expressed component. These cases of double negative transcriptional control results in an indirect “positive” transcriptional control by the clock component expressed in daytime before the respective component. However, there is empiric evidence that supports the direct positive regulation of PRRs by LHY/CCA1 (Pokhilko et al. 2012). Main components of the central oscillator: CCA1 – CIRCADIAN CLOCK ASSOCIATED 1. LHY – LATE ELONGATED HYPOCOTYL. PRR 9, 7 and 5 – PSEUDO RESPONSE REGULATOR 9, 7 and 5. EC – evening complex. LUX – LUX ARRHYTHMO. ELF3 and 4 – EARLY FLOWERING 3 and 4.
(Continued from page 10.) Outputs of the plant circadian clock are manifold, including plant growth and development, photoperiodism and anticipatory physiological preparation for daily repeating abiotic and biotic stressors (see text for examples). The figure was re-drawn from figure 3a in Harmer (2009) and the simplified clock model is re-drawn from figure 8D in Pokhilko et al. (2012). Descriptions are also from these references.

The linear clock model presented in Fig. 2 is oversimplified insofar as findings in recent years suggest that the clock is rather a complex network than a linear structure where all three components (inputs, oscillator and outputs) can feedback on each other; e.g. even inputs can directly feed-back on output pathways, and vice versa (Harmer 2009, Pruneda-Paz and Kay 2010). Another way this representation is too simple is the fact that, while the model only depicts transcriptional control, post-transcriptional and post-translational regulation and chromatin remodeling are also important mechanisms in the oscillator (Nagel and Kay 2012).

When entraining to longer or shorter days, plants must adjust the expression of their clock components according to their environment for correct anticipation of dawn. Dawn and dusk have been indicated as important time-points for this resetting of the clock (Roenneberg and Foster 1997); increased sensitivity of the clock at these times is achieved by the phenomenon known as “gating”, in which the clock is less sensitive to external stimuli at less informative times of day, and more sensitive at more informative times (McWatters and Devlin 2011).

Processes that facilitate adjustments to changing photoperiod might be similar to those that lead to re-adaptation of plants to a new L/D regime after transfer, the recovery from a “jet-lag” (Roenneberg and Foster 1997); however, the difference is that of acclimatizing to a gradual change in day length and a situation in which a rapid re-adaptation of the oscillator is necessary. According to the current theoretical model, presented in simplified version in Fig. 2, both morning and evening loop contain elements whose expression or protein levels are light-responsive. However, the acute activation of CCA1 and LHY (CIRCADIAN CLOCK ASSOCIATED 1; LATE ELONGATED HYPOCHOTYL) gene expression by light is predicted to be mainly responsible for delays and advances in the phase of the clock, induced by light-input from the environment (Pokhilko et al. 2012).
The circadian clock runs in every cell of the plant, but, in contrast to animals, these individual cell clocks are not entrained by a central master clock (Koukkari and Sothern 2006). For example, tissues of the same plant have been successfully entrained to different rhythms (Thain et al. 2000) and cells of the same tissue can become desynchronized under constant environmental conditions (Wenden et al. 2012). On the other hand, roots might depend on signals from the shoot for appropriate entrainment to diurnal conditions (James et al. 2008). This is why entrainment cues like light might have special importance for synchronizing plant clocks on the whole-plant level (McWatters and Devlin 2011).

The plant clock might have an independent evolutionary origin, because hardly any components of the plant clock are shared with clocks in other eukaryotic organisms (McWatters and Devlin 2011). On the other hand, there has been considerable evolutionary conservation of clock components in angiosperms, as genes homologous to *A. thaliana* clock genes could be identified in many species, both monocots and dicots (McClung 2013). Specifically, in the wild tobacco *Nicotiana attenuata* clock gene homologues NaLHY, NaTOC1 and NaZTL could be identified (Yon et al. 2012) and *N. attenuata* above and belowground tissues show a rich pattern in diurnal primary and secondary metabolite oscillations, before and after simulated herbivory (Kim et al. 2011). Furthermore, flower movement appears to be a circadian clock-controlled trait in *N. attenuata* (Yon et al., unpublished data).

### 1.3. Evidence for fitness advantages conferred by a functioning circadian clock?

Plants have been shown to gain a fitness advantage from having a circadian clock appropriately entrained to the external environment (Green et al. 2002, Michael et al. 2003, Dodd et al. 2005, Yerushalmi and Green 2009). Critically, entrainment is what allows the clock to synchronize with relevant diurnal and seasonal events in an organism’s environment. Furthermore, plant responses to abiotic stress (e.g. Espinoza et al. 2008, Wilkins et al. 2010, Sanchez et al. 2011, Seo et al. 2012) and to the biotic stress of biotrophic pathogen attack (Bhardwaj et al. 2011, Wang et al. 2011, Zhang et al. 2013) have been shown to be mediated by the circadian clock.
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Recent findings indicate that the circadian clock might even be involved in the regulation of direct defense against insect herbivores (Goodspeed et al. 2012, 2013a, b). There are numerous reasons why herbivores are able to entrain to light/dark regimes: The molecular mechanism of the insect clock has been elucidated in *Drosophila melanogaster* and homologues of these clock genes and light-sensing components have been identified in many other insect species, among them a number of lepidopterans (Tomioka and Matsumoto 2010). Moreover, appropriate time-keeping and perception of day length are also likely to be of fitness advantage to insects, as life histories of many insect species are shaped by photoperiodic events such as entry into dormancy (Koštál 2011).

Recently, Goodspeed *et al.* (2012) reported that, under diurnal and constant conditions, larvae of the lepidopteran generalist *Trichoplusia ni* showed a circadian feeding pattern on artificial diet, with a maximum toward the end of the actual or subjective day. Furthermore, results from a circadian experiment showed that this predictable behavior might have ecological consequences in a plant-herbivore pair and that proper entrainment with the environment can confer a higher level of plant resistance. *T. ni* larvae were left to infest *A. thaliana* wild-type (WT) plants for 72h under constant conditions (constant light, LL, or constant dark, DD), after plants and larvae had been entrained under the same 12 h:12 h light:dark cycle (in-phase) or under opposite 12h:12h light:dark cycles, with a phase difference of 12h (out-of-phase). Larvae on plants that received the opposite entrainment gained more mass and removed more leaf area from their host plants than did larvae on host plants with the same entrainment regime. The authors speculated that this apparent higher plant resistance, mediated by synchronized entrainment in a plant-herbivore pair, might be attributed to a putative mechanism for anticipation of attack in *A. thaliana* plants, as constitutive and *T. ni* herbivory-induced jasmonate levels fluctuated in a circadian manner and reached their peak accumulation in the middle of the actual or subjective day, in advance to the observed peak of *T. ni* feeding. However, besides this no potential biochemical background for this phenomenon is detailed, although the authors could conclusively show that lack of a functioning circadian clock in arrhythmic *A. thaliana* mutant lines (*CCA1* overexpression, *CCA1-OX*, and the *LUX ARRHYTHMO* knockout *lux2*), or impaired jasmonate biosynthesis in *A. thaliana* knockout lines (*allene oxide synthase, aos*, and *jasmonate resistant 1, jar1*)
abolished the fitness benefit of synchronous entrainment relative to asynchronously entrained plants (Goodspeed et al. 2012).

Despite the obvious holes in the biochemical background of this observed phenomenon, another level in this putative circadian fluctuation of attack anticipation might have been uncovered when Shin and colleagues (2012) showed that MYC2, a transcription factor which mediates jasmonate signaling, oscillated in transcript as well as protein levels throughout the day with a maximum toward dusk, in accordance with the circadian oscillation of constitutive and induced jasmonate levels in *A. thaliana* and times of presumed maximum feeding activity in *T. ni*. Moreover, in a more recent follow-up study, Goodspeed and co-workers (2013b) were able to demonstrate similar effects for harvested fruits and vegetables from several plant families, entrained under 12 h:12 h light:dark (L/D) cycles prior to, or during infestation by *T. ni*.

The aim of this thesis is the question whether plant resistance is generally reduced when plants and herbivores are entrained out-of-phase with each other. An experimental design similar to that of Goodspeed *et al.* (2012) was used to test for this effect on a co-evolved plant-herbivore pair: the model plant *Nicotiana attenuata* Torr. ex. Watson (Solanaceae) attacked by its native specialist herbivore *Manduca sexta* Linne (Sphingidae).

1.4. Recognition of the herbivore specialist *Manduca sexta* by *Nicotiana attenuata and associated defense responses*

*N. attenuata* is an annual native to the Great Basin Desert of the southwestern USA (Baldwin 1989) (**Fig. 3A**). It has a short generation time and uses the ephemeral growth strategy of germinating after wildfires from long-lived seedbanks into an environment with little inter-specific, but high intra-specific competition. It interacts with various herbivore species with a set of inducible defensive traits (Baldwin et al. 2001). Generally, *N. attenuata* shows little tolerance for shading (Fragoso V., unpublished data). *M. sexta* larvae (**Fig. 3 B**) attack the plant’s leaves, but the
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**Figure 3: Nicotiana attenuata and Manduca sexta in their native habitat.**

A) *N. attenuata* plant flowering. B) *M. sexta* larva, end of 4th instar, feeding on *N. attenuata*. C) *M. sexta* adult moths mating. Photo A was taken by Celia Diezel, and photos B and C were taken by Danny Kessler. The photos are used with their permission.

nocturnal adults (Fig. 3C), among them ovipositing females, are important pollinators (Kessler and Baldwin 2001, Kessler et al. 2008).

*M. sexta* herbivory induces the accumulation of a number of secondary metabolites with a known physiological effect on *M. sexta* performance, in leaf tissue of *N. attenuata* leaf rosettes, and the plant’s reaction to this herbivore is a highly specific response, mediated by certain herbivory associated elicitors that plants are able to detect, deposited in the disrupted tissue during feeding (Bonaventure 2012). The main active compounds in *M. sexta* oral secretions are so-called fatty acid-amino acid conjugates (FACs), mainly N-linolenoyl-glutamic acid (18:3-Glu) (Alborn...
1997, VanDoorn et al. 2010), but the concrete receptor for compounds in saliva still remains unknown (Baldwin et al. 2001, Bonaventure 2012). Perception of FACs in *M. sexta* oral secretions activates a signaling pathway via a cascade of protein kinases which, by means of an unknown mechanism, play a role in activating the jasmonic acid production, via the oxylipin pathway in chloroplasts and subsequent steps in peroxisomes (Wasternack 2007, Wasternack and Hause 2013). This protein kinase cascade also brings about a transient burst in ethylene accumulation (Bonaventure 2012).

Temporarily increased jasmonate levels induce the activation of a number of transcription factors (TFs) in the plant cell nucleus and thus the activation of jasmonate-induced defense pathways (Kaur et al. 2010, Onkokesung et al. 2012, Woldemariam et al. 2013). Subsequently, *N. attenuata* accumulates a variety of defense compounds in shoot tissue in response to *M. sexta* feeding, e.g. shoot-synthesized hydroxygeranyllinalool-diterpene glycosides (HGL-DTGs) and phenylpropanoid – polyamine conjugates (PPCs) (Heiling et al. 2010, Kaur et al. 2010, Onkokesung et al. 2012). After herbivory, there are also higher accumulations of the alkaloid nicotine in *N. attenuata* tissue; this compound is both an important constitutive and induced defense compound against generalist herbivores in *N. attenuata*, but is less effective against *M. sexta* larvae, and accumulation is attenuated after *M. sexta* damage (Steppuhn et al. 2004, von Dahl et al. 2007). But, although *M. sexta* is tolerant to high levels of nicotine, its growth is reduced on nicotine-containing compared to nicotine-deficient plants (Steppuhn et al. 2004). While after events of mechanical leaf wounding, nicotine biosynthesis in roots increases and more nicotine is allocated to the site of wounding in the shoot, significantly above constitutive levels, in the specific defense response to *M. sexta* herbivory, this nicotine accumulation is attenuated by the ethylene burst (Kahl et al. 2000, Baldwin et al. 2001).

In the interaction between *M. sexta* and *N. attenuata*, it has been shown that not only secondary, but also primary metabolism is reconfigured by attack (e.g. Halitschke et al. 2001, Schwachtje et al. 2006, Machado et al. 2013).
1.5. Aim of the experimental work

In this thesis, I aim to investigate the interaction in the plant-herbivore pair *N. attenuata*-*M. sexta* for a similar effect as demonstrated by Goodspeed *et al.* (2012) in the *A. thaliana*-*T. ni* pair. One important difference is that *M. sexta* is a specialist on *N. attenuata* (Halitschke et al. 2001), while *T. ni* is a generalist on *A. thaliana* (Goodspeed et al. 2012) and, therefore, we actually do not only test whether plant resistance is generally reduced when plants and herbivores are entrained out-of-phase to each other, but also whether this effect applies to specialist herbivores. Jander (2012) pointed out that it is evolutionarily unexpected to observe such a specific tailored response to a generalist’s activity, and especially to a generalist that is probably not encountered regularly in the field by *A. thaliana*. An argument of applicability to expected attack patterns of other day-active herbivores (Jander 2012) is problematic, because herbivores have multiple activity patterns; for instance, predominantly night-active slugs and snails have been shown to feed on *A. thaliana* (Falk et al. 2013).

Another advantage of the model organism *N. attenuata* is that its defense responses have been subject to years of research (Schuman and Baldwin 2012) and direct defenses with physiological activity against the specialist *M. sexta* have been identified (see section 1.4). This gave me the opportunity for targeted analysis of putative differences in plant resistance between in-phase and out-of-phase entrained plants; specifically HGL-DTGs, the phenylpropanoid–polyamine conjugate caffeoylputrescine (CP) and nicotine were quantified before and after *M. sexta* feeding for all experimental set-ups. To gain a broader overview of the general responses of plants under various light conditions, because both primary and secondary metabolism are known to be involved in these induced defense responses (e.g. Halitschke et al. 2001, Schwachtje et al. 2006, Machado et al. 2013), I also generated untargeted general metabolite profiles of the attacked tissues (Gaquerel et al. 2010, Kim et al. 2011).

Constant light (LL) and constant dark (DD) conditions can both have detrimental effects on plant physiology: e.g. photo-toxicity symptoms in LL (see Sysoeva et al. 2010 for a review), or carbon starvation in DD (e.g. Graf et al. 2010) and I
considered that these additional stressors might impact induced defense responses negatively. This is why another scenario was added to the LL and DD conditions employed by Goodspeed et al. (2012). “Jet-lagged” plants were generated by subjecting them to herbivory under a diurnal regime (LD), specifically the in-phase regime, after plant entrainment in the opposite LD regime, specifically the out-of-phase regime; it was presumed that host plants were transiently out-of-phase entrained relative to feeding caterpillars. For the jet-lagged plants, I expected attenuated or slower induced defense responses; but these would be due to the stress of miss-matching physiological processes with the new light regime rather than due to the stress of constant exposure to light or a lack of light. Along this line of thinking, it was decided to quantify the starch and soluble sugar content of leaf material as a starvation indicator (in DD) and as an indicator for photosynthetic activity (in LD and LL).

One of the points of criticism on the experimental design of Goodspeed et al. (2012) was that the observation of a circadian pattern of feeding activity in T. ni larvae had not been done on host plants, but only on artificial diet (Jander 2012); however, feeding behavior of insect herbivores can potentially be modified by their own host plants (Shiojiri et al. 2006). Since quantifying removed leaf area directly on plants in a time-series proved problematic in preliminary experiments, I employed a middle-way by quantifying removed leaf area, as a putative plant perceived damage cue per time-intervals, while M. sexta larvae fed on excised leaves. Since prior studies have shown M. sexta larval feeding rate to be strongly correlated with temperature (e.g. Kingsolver and Woods 1997, Petersen et al. 2000), it was considered that controlled conditions in the climate chambers might not be sufficient to bring about predictive rhythmic behaviour in caterpillar feeding; moreover, since M. sexta larvae grow rapidly, (see section 3.1. and supplemental table 2), it was not unreasonable to expect a rapid increase in feeding rates and damage as caterpillars grow.

Finally, there are probably limits to comparisons between effects encountered in the A. thaliana-T. ni plant-herbivore pair and those encountered in N. attenuata infested by M. sexta, as N. attenuata plants rely heavily on their induced defense responses (Steppuhn et al. 2004, Gális et al. 2010, Heiling et al. 2010, Kaur et al. 2010) and it is not quite clear how a constitutive circadian oscillation in defense anticipation
would shape the accumulation of secondary metabolites that is observed after simulated herbivory (= mechanical damage plus application of *M. sexta* oral secretions) over several days (see e.g. Gális et al. 2010 for caffeoylputrescine accumulation).
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2.1. Cultivation of Nicotiana attenuata wild-type plants

All experiments were performed with *N. attenuata* Torr. Ex. Wats. (Solanaceae) wild-type (WT) plants from a 31st-generation inbred line originating from seeds collected at the Desert Inn Ranch in Washington County, Utah, USA in 1988 (Glawe et al. 2003). Germination proceeded as previously described by Krügel et al. (2002). Briefly, *N. attenuata* seeds were surface-sterilized for 5 min in 5 mL aqueous solution of 0.1 g dichloroisocyanuric acid (DCCA; Sigma-Aldrich, St. Louis, MO, USA), supplemented with 50 μL of 0.5% (v/v) Tween-20 detergent (Merck, Darmstadt, Germany). After washing three times with deionized, autoclaved water, seeds were incubated for 60 min in 5 mL sterile liquid smoke (House of Herbs, Inc., Passaic, New Jersey, USA) solution, 50 times-diluted in water and containing 50 μL of 0.1 M gibberellic acid (GA3; Carl Roth GmbH + Co. KG, Karlsruhe, Germany). After washing three times with deionized, autoclaved water, seeds were incubated for 60 min in 5 mL sterile liquid smoke (House of Herbs, Inc., Passaic, New Jersey, USA) solution, 50 times-diluted in water and containing 50 μL of 0.1 M gibberellic acid (GA3; Carl Roth GmbH + Co. KG, Karlsruhe, Germany). After repeating the washing procedure, 20-30 seeds were placed on a petri dish containing germination medium (Gamborg’s B5 medium with minimal organics (Sigma-Aldrich) and 0.6% (w/v) phytagel (Sigma-Aldrich)), utilizing a glass Pasteur pipette (Carl-Roth GmbH). Petridishes were kept under long-day conditions (16 h light : 8 h dark) in a growth chamber (at 26°C and 155 μmol m⁻² s⁻¹ in light, and at 24 °C in dark; Percival, Perry Iowa, USA) until 10 days after germination, and then moved to small pots (TEKU JP 3050 104 pots; Pöppelmann GmbH & Co. KG, Lohne, Germany) with Klassmann plug soil (Klassmann-Deilmann GmbH, Geesten, Germany).

Plants were cultivated in the glasshouse for another 10 days under growth conditions as described in Krügel et al. (2002) with a day/night cycle of 16 h/8 h, at 1000-1300 μmol m⁻² s⁻¹ photosynthetic photon flux density - under daylight plus supplemental light by 450 W Na-vapor high-intensity discharge bulbs (Master Sun-T PIA Agro 400 or Master Sun-T PIA Plus 600-W sodium lights; Philips, Amsterdam, Netherlands). Air temperatures in the glass house were 23°C to 25°C during lights-on and 19°C to 23°C during lights-off; relative humidity was 45%–55%. Plants were supplied daily with 0.5 g/L Peters Allrounder fertilizer (Scotts Miracle-Gro
Company, Marysville, Ohio, USA) containing nitrate, potassium and magnesium + 0.1 g/L Borax.

Twenty days after germination treatment, individual small rosette plants were transplanted to 1 L plastic pots containing Frühsdorfer Nulldre (HAWITA Gruppe GmbH, Vechta, Germany) with 0.5 g/L PG mix (Yara International ASA, Oslo, Norway), 0.9 g/L Ca(H2PO4)2, 0.35 g/L MgSO4 * 7 H2O (Sigma-Aldrich), and 0.055 g/L Micro Max (Everris International B.V., Geldermalsen, Netherlands). After repotting, plants for the *M. sexta* performance experiments were cultivated in two climate chambers (Johnson Controls Unitary Products, Norman, OK, USA; dimensions: 4.00 m length, 2.22 m width and 2.35 m height), for at least another 10 days. Plants were cultivated under an either 12 h light: 12 h dark or 12 h dark: 12 h light regime, 65% humidity and 24 °C air temperature. In climate chambers, light was provided by Master Sun-T PIA Agro 400 W (Philips) sodium lamps, at 100% intensity setting. Plants were grouped in batches of 5 plants, each batch on an individual tray (Manna-Pikierschale Nr. 956, size: 60 x 40 x 6.4 cm; H. Nitsch & Sohn GmbH & Co. KG, Kreuztal, Germany), to facilitate watering. Throughout experiments, plants were watered 6 d/week; at the first day, with 200 mL per plant, and from then on with 100 mL per plant. Peter’s Professional Allrounder 20-20-20 N-P-K+TE fertilizer (Everris International B.V.) was added once per week to the water, at a concentration of 0.75 g/L.

Plants were used for the larval performance experiments under constant dark (DD) or constant light (LL) conditions and under diurnal (LD; 12 h:12 h light:dark) conditions. For the 24h assays of feeding activity, under various light conditions, plant material was collected from *N. attenuata* rosette stage plants, cultivated under similar conditions and a 12h light: 12h dark diurnal regime.

2.2. Rearing of *Manduca sexta* larvae

*Manduca sexta* Linne eggs originated from an in-house colony at the Max Planck Institute for Chemical Ecology in Jena. For performance assays, eggs were incubated under the in-phase entrainment conditions (12 h light : 12 h dark) in the climate
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chamber for at least 2 days, and for an additional 1 day under either LL or DD, in experiments under constant conditions (Fig. 4). From prior experience, egg collection and incubation were timed so, that major hatching occurred within the last 12 h of incubation. At the end of incubation, *M. sexta* neonates were placed on the plants, utilizing forceps. Larvae hatched within up to 5 days after oviposition.

Figure 4: Experimental designs for caterpillar performance experiments under constant conditions (a) or diurnal conditions (b). Boxes represent 12 h intervals of either light (white squares) or dark (black squares); the change from diurnal to constant conditions (constant light, LL or constant dark, DD) is indicated by the change to grey squares. Angled arrows from above show time points at which plant material was collected (before transfer to constant conditions, before and 3 d after infestation by *M. sexta* larvae); arrows from below show when *M. sexta* larvae were transferred to or from plants. *M. sexta* infestation lasted for 3 d, after which larvae were placed on new plants of the same treatment group. After day 3, transfer to new plants occurred every 2 d (not depicted; see methods section 2.3). Representation of the experimental design was adapted from Goodspeed *et al.* (2012).
2.3. Caterpillar performance experiments

In this experimental set-up I assessed the development of caterpillar mass, as *M. sexta* fed on *N. attenuata* plants of different entrainment and interpreted this variable as a direct indicator of caterpillar performance and an indirect indicator of plant resistance (Zavala et al. 2004).

*N. attenuata* rosette plants were grown in two climate chambers (Johnson Controls Unitary Products) for at least 10 days after transfer from the greenhouse (see section 2.1. for prior treatment). The switch from night to day was at 9 am in one chamber and at 9 pm in the other chamber; thus, plants were entrained in either light/dark 12 h:12 h or dark/light 12 h:12 h. When *N. attenuata* plants enter the flowering stage, herbivory-induced defense responses are reduced (Diezel et al. 2011), but small leaf rosettes would not have provided enough food for *M. sexta* larvae. Therefore, I used plants that were late rosette to early elongation stage. Similar caterpillar performance experiments were conducted under diurnal conditions (8th to 17th August, 2012) and under constant conditions (LL and DD conducted simultaneously; 9th to 19th December 2012; starting with the transfer of plants and eggs to LL or DD, respectively).

For the performance experiment under diurnal conditions (see Fig. 4b), at 9 am, on the first day of the experiment, 10 plants were transferred from dark/light 12h:12h to light/dark 12h:12h. Recently, hatched *M. sexta* larvae were placed on *N. attenuata* WT plants, 6 neonates on three fully developed rosette leaves per plant, on 10 plants per treatment in light/dark 12h:12h. Treatments were “in-phase” (= control treatment: both plants and larvae had been entrained under light/dark 12h:12h), and “out-of-phase” (= transferred treatment: larvae were entrained in light/dark 12h:12h, while plants were entrained under dark/light 12h:12h). I assumed that 48 hours incubation were enough for caterpillars to entrain to the phase of the diurnal regime presented to them in the climate chamber. We also assumed that plants of the “out-of-phase” treatment would take several days to re-entrain to the new light regime. Damaged plants in each treatment were exchanged for undamaged plants of the same entrainment batch, after 3 days of larval feeding - the first time-interval; in the case of “out-of-phase” plants, these were exchanged for plants of the same entrainment batch newly transferred from dark/light 12h:12h. At the first exchange...
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of plants, three caterpillars, if possible from the same damaged plant, were placed together on a new plant of the respective treatment, resulting in 2-3 caterpillars per plant on up to 20 plants of each treatment. Due to mortality in early instars, decreases in caterpillar numbers in both treatments were to be expected. Furthermore, it was necessary to redistribute some caterpillars between plants at times of plant exchange in later time-intervals, since caterpillar movement between five plants of the same treatment on an individual tray was facilitated by proximity as well as overlapping rosette leaves between plants. Specifically for this reason, all caterpillars were treated as biological replicates of caterpillar growth, not quantified separately by the factor of host plant. In subsequent exchanges of new host plants, if possible, up to 3 caterpillars from one plant were placed together on a new plant. *M. sexta* infestation lasted 3 days for the first time interval, until plants were exchanged, but was reduced to 2 days for the second, third and fourth time interval. This was due to the greater damage to plants, as later instar larvae would have defoliated plants completely within three days. At the second exchange of host plants and at all remaining exchanges, the caterpillars were weighed. (I began weighing larvae in the second instar, because first instar larvae have a higher mortality from handling.) Mass was determined to ± 0.001 g deviation with a balance (Sartorius AG, Göttingen, Germany). The experiment was terminated when the biggest caterpillars (end 4th to early 5th instar) were now actively looking for plants outside their assigned trays.

For the experimental set-ups **under constant dark and constant light** (Fig. 4a), at 9 am, 24h before *M. sexta* infestation, “in-phase” and “out-of-phase” plants were transferred from their prior entrainment regime to the respective constant conditions (LL or DD), five plants for each treatment under constant conditions for the first time-interval. Simultaneously, egg boxes were transferred to constant conditions. (The point of this pre-treatment was to abolish metabolic differences between plants of both treatments, e.g. differences in food quality due to “end of day” vs. “end of night” conditions, as seen in Fig. 4, and to avoid possible transitory cycles as they can occur in the first time of transition to constant conditions (Koukkari and Sothern 2006).) After this 24h pre-treatment, freshly hatched *M. sexta* larvae were placed on fully developed rosette leaves of *N. attenuata* WT plants, on five plants per treatment. Specifically, 9 neonates were distributed between three leaves in each
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plant. After 3 days of larval feeding, host plants in each treatment were exchanged for undamaged plants, of the same entrainment batch and 24h pre-treatment in constant conditions. At the first exchange of host plants, 4-5 caterpillars, if possible from the same damaged plant, were placed together on a new plant of the respective treatment, resulting in 4-5 caterpillars per plant on ten plants of each treatment. In subsequent exchanges of new host plants, due to mortality, 3-4 caterpillars, if possible, from one plant were placed together on a new plant. Again, *M. sexta* infestation lasted 3 days for the first time interval, but was reduced to 2 days for the second, third and fourth time interval to ensure sufficient plant material. At the second exchange of host plants and at all remaining exchanges, the caterpillars were weighed, as described for diurnal conditions.

The constant conditions were achieved using two climate chambers set to opposite entrainment regimes, by transferring plants and *M. sexta* egg boxes in pre-treatment, or infested plants, respectively, between chambers every 12h. Thus, organisms, under constant dark, would be exposed to the lights-off phase in both chamber, and under constant light, would be exposed to the lights-on phase in both chambers. For pre-treatment and *M. sexta* infestation in DD, transfer without exposure to light was achieved by packaging organisms in lightproof containers at the end of the 12h dark phase and then transferring them to the other climate chamber, after lights-off. Three green LED lights (each 1 W, \( \lambda = 520 \) nm) were used for illumination of entire chambers during experimental measurements under DD, and curtains were hung in front of chamber doors to avoid accidental light exposure from outside. For constant light, organisms were carried unpackaged between chambers.

Caterpillar mass data was processed with Microsoft Excel 2010 (Microsoft Corporation, Redmond, WA, USA). Average caterpillar mass at a time point was calculated from the mass of all caterpillars (biological replicates) of one treatment.
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Figure 5: Phyllotactic numbering in the mature leaf rosette of a 31 day old *N. attenuata* plant. *N. attenuata* rosette leaves grow in a spiral around the rosette axis and, when a particular leaf is considered, another leaf one position younger or older is usually ~120° degrees removed in the spiral. Numbering of leaves is respective to the source-sink transition leaf (position 0), which has just attained full expansion and imports and exports similar amounts of carbon from photosynthesis. Sink leaves are those leaves where the balance between these two processes is tipped toward importing carbon resources for growth (position -1 and younger); in source leaves, the balance is tipped toward exporting more carbon resources than are imported (position +1 and older). Descriptions are from Giri et al. (2006). Representation was adapted from Giri et al. (2006). The photo was taken by Jasmin Herden. Scale: 13.6 cm pot diameter, from edge to edge.

2.4. Collecting of plant material

Instead of remaining leaf area (as in Goodspeed et al. (2012)), I quantified a more direct, biochemical indicator of plant resistance, the general metabolomics response in attacked leaf tissue after 3 days herbivore infestation (see methods section 2.6 for metabolomics analysis via UPLC-ESI-MicroToF). Relative separation of general metabolomics profiles before and after herbivory is usually a good indicator of the induced defense response of *N. attenuata* and thus, plant resistance (see e.g.
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Gaquerel et al. (2010), Kim et al. (2011). Furthermore, a number of target metabolites were quantified (see introduction section 1.4. for reasons in choosing these target metabolites and methods section 2.7 for quantification of target secondary metabolites via HPLC).

Before the caterpillar performance experiments commenced, I determined relative leaf positions within the *N. attenuata* rosettes (Fig. 5), in order to collect leaf material of a similar age and similar sink-source dynamics.

During the first time-interval, for diurnal conditions, i.) one fully developed rosette leaf was collected from each *N. attenuata* plant before I placed caterpillars, and ii.) the most heavily attacked leaf or leaves of a plant were collected after herbivory. For diurnal conditions, leaf material was collected from 8 biological replicates.

Similarly, for constant conditions, plant material was collected during the first time-interval, at three time-points, for each treatment: i.) before 24h pre-treatment in constant conditions (leaf position 0), ii.) before (leaf position + 1) and, iii.) after 3 days *M. sexta* infestation in constant conditions (leaf positions + 3 to + 6; most heavily attacked leaf or leaves). For constant conditions, leaf material was collected from 5 biological replicates.

Leaf mid-veins are usually not consumed by young larvae and were removed at collection. The remaining leaf tissue was immediately flash-frozen in liquid nitrogen and subsequently stored at -80 °C until extraction.

Plants were treated as biological replicates for measures of plant metabolites. Within the first time-interval, material was repeatedly sampled from the same plants in a treatment due to the limited number of plants that fit into the climate chambers, limiting the possibilities for statistical analyses. Plants were not given numbers, making repeated measurement tests in statistical analysis impossible.

2.5. Extraction procedure for plant metabolites

Frozen leaf material, previously flash-frozen in liquid nitrogen and stored at -80 °C, was manually ground to a fine powder with a plastic pestle in 2 mL microcentrifuge tubes (Eppendorf International, Hamburg, Germany). Per sample, around 55 mg of the ground leaf tissue were weighed into another 2 mL microcentrifuge tube (±
0.0001 g deviation) with a balance (Sartorius AG) and the exact masses were recorded.

Extraction of metabolites was performed as described in Gaquerel et al. (2010): 500 μL of extraction buffer (40% methanol (HPLC grade; VWR International Ltd., Leicestershire, England), v/v, containing 40 mM acetic acid (Merck) and 44 mM ammonium acetate (Sigma-Aldrich); pH 4.8) were added to the frozen, ground leaf tissue in each sample tube. After adding two steel balls to each tube, samples were homogenized twice using Genogrider 2000 (SPEX SamplePrep, Metuchen, NJ, USA) at 250 strokes per min for 45 seconds. To remove particles, supernatants were collected after three time of sequential centrifuging at 16,100 g for 20 min at 4°C. 200 μl of the supernatant were transferred to a HPLC crimp vial N11-1 HP with a micro insert of 6 x 31 mm (Macherey-Nagel, Düren, Germany) for measurement of untargeted metabolite profiles (see section 2.7.) and quantification of target secondary metabolites (see section 2.6.). Pooled aliquots from all treatment groups were used as quality control to monitor instrument stability during untargeted metabolite analysis (see section 2.7.); extraction buffer served as blank for all metabolomics and target secondary metabolite measurements (see sections 2.6. and 2.7.).
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(See next page for figure caption.)
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**Figure 6: Examples for induced responses in *N. attenuata* under attack by *M. sexta.*

Feeding induces a transient, burst-like increase in phytohormone levels of jasmonates and ethylene. In *N. attenuata* leaf tissue, *M. sexta* herbivory induces the increased biosynthesis of HGL-DTGs (Heiling et al. 2010) and the phenylpropanoid – polyamine conjugate (PPC) caffeoylputrescine (e.g. Kaur et al. 2010). After events of mechanical leaf wounding, nicotine biosynthesis in roots increases and more nicotine is allocated to the site of wounding in the shoot, significantly above constitutive levels. But in the specific defense response to *M. sexta* herbivory, this nicotine accumulation is modified by the ethylene burst, specifically the accumulation is attenuated (Kahl et al. 2000, Baldwin et al. 2001). But at the same time, other phenolic compounds, chlorogenic acid and rutin are in general not induced upon *M. sexta* herbivory (Keinänen et al. 2001, Kaur et al. 2010). Scheme is based on Baldwin *et al.* (2001) and Bonaventure (2012). Structural formulas for caffeoylputrescine, chlorogenic acid and rutin are from (Kaur et al. 2010). The exemplary structure formula of the simple HGL-DTG lyciumoside was based information in Heiling *et al.* (2010) and Gaquerel *et al.* (2010). The structure formula of nicotine is from Taiz and Zeiger (2006).

2.6. **Quantification of target secondary metabolites via high performance liquid chromatography**

This method was employed to quantify the target secondary metabolites nicotine, caffeoylputrescine (CP), chlorogenic acid (CGA), rutin and hydroxygeranyllinalool diterpene glycosides (HGL-DTGs) (see Fig. 6 for the chemical structure). They show different reactions to *M. sexta* attack, ranging from strong induction in HGL-DTGs and CP, to attenuated accumulation in nicotine and no induction in rutin and CGA (see Fig. 6). The latter two compounds might work as a kind of control as they should not increase with herbivory and might indicate negative or positive effects of different light regimes on general plant physiology.

Measurement followed the method developed by Oh *et al.* (2012). Briefly, one µL of extract was analysed by high pressure liquid chromatography (HPLC 1100 series; Agilent, Böblingen, Deutschland), using a C18 column (Chromolith FastGradient RP18e column, 50 * 2 mm internal diameter, monolithic silica with bimodal pore structure, macropores with 1.6 µm diameter; Merck) equipped with a C18 precolumn (Gemini NX RP18, 3 µm particle size, 2 x 4.6 mm internal diameter; Phenomenex International, Torrance, CA, USA), with a column oven set at 40°C. The mobile phase comprised solvent A (0.1 % formic acid (Sigma-Aldrich) and 0.1 %
ammonium hydroxide (Sigma-Aldrich) solution in deionized water (pH 3.5)) and solvent B (methanol, HPLC grade; VWR International Ltd.), used in a gradient mode (0 - 0.5 min isocratic at 0% solvent B, 0.5 – 6.5 min linear gradient to 80% solvent B, 6.5 - 10 min at 80% solvent B, and 10 – 15 min re-equalibration to 0% solvent B) with a flow rate 0.8 mL/min. Separated analytes were detected with PDA (Photo Diode Array) and ELSD (Evaporative Light Scattering Detector, Varian) detectors. The retention times of target compounds were similar to the retention times given by Oh et al. (2012): According to their paper, nicotine should elute at a retention time (RT) of ~0.5 min (detected by UV absorbance at 260 nm); CP generally elutes at a RT of ~2.6 min and the two isomers of CGA elute at ~2.9 and ~3.2 min, respectively (detected at 320 nm); rutin should elute at RT 4.7 min and was detected at 360 nm. Diterpene glycosides peak pool, eluting between RT 7.0 and 8.5 was detected by ELSD detector.

The peak areas were integrated using the Chromelone chromatographic software (version 6.8; Dionex Corporation, Sunnyvale, USA). The amount of nicotine, CGA, and rutin in plant tissue was calculated using external standard curves (7.8, 15.6, 31.3, 62.5, 125 and 250 µg/mL of each standard). CP content was estimated based on the external CGA calibrations and expressed as CGA equivalents. HGL-DTGs content was expressed as relative peak area. Concentration values below the range of the standard curves were expressed as not quantifiable. The amount of metabolites in the plant tissue was expressed as amount per g fresh mass of the leaf material.
2.7. Measurement of untargeted metabolite profiles with UPLC/ESI-TOF-MS

Samples were measured with reversed phase, ultra-high pressure liquid chromatography – electrospray ionization – time-of-flight mass spectrometry (UPLC/ESI-TOF-MS; Bruker Corporation, Billerica, MA, USA) as described in Gaquerel et al. (2010) with modifications as detailed in Kim et al. (2011). Two µL of the leaf extract were separated using a UPLC system (UltiMate 3000 Rapid Separation; Dionex Corporation). Separation was done on a reverse-phase C18 column (150 mm * 2.1 mm inner diameter, 2.2 µm particle size; Acclaim, Dionex Corporation) using a binary solvent gradient: Solvent A (deionized water containing 0.1% [v/v] acetonitrile (HPLC grade; VWR International Ltd.), and 0.05% formic acid (Sigma-Aldrich)), solvent B (acetonitrile (VWR International Ltd.) and 0.05% (v/v) formic acid (Sigma-Aldrich)). The following gradient conditions were used for the chromatography: 0 – 1 min isocratic at 10% solvent B, 1 – 9 min linear gradient to 80% solvent B, 9 - 11 min isocratic at 80% solvent B and re-equilibration to 10% solvent B for 4 min. The flow rate was 300 µL/min. Eluted compounds were detected by a MicroToF mass spectrometer (Bruker Corporation) using electrospray ionization in positive mode. Typical instrument settings were as follows: capillary voltage, 4500V; capillary exit voltage, 130 V; drying gas (200°C) at 8 L/min. Detected ion range was from mass-to-charge ratio (m/z) 200 to 1400 and mass spectra were recorded with a repetition rate of 1 Hz. Internal mass calibration was achieved using a sodium formate solution (10 mM sodium hydroxide (Carl-Roth GmbH) and 0.2% formic acid (Sigma-Aldrich) in isopropanol (Merck)/water 1:1, v/v).

Raw data was processed as described in Gaquerel et al. (2010) and Kim et al. (2011). Raw data files were converted to the netCDF format using the export function of the Data Analysis v4.0 software (Bruker Corporation) and processed using the XCMS R package (e.g. Gaquerel et al. 2010, Kim et al. 2011) (http://fiehnlab.ucdavis.edu/staff/kind/Metabolomics/Peak_Alignment/xcms/). Peak detection was performed using the centWave algorithm and the parameter settings ppm=20, snthresh=10, peakwidth=5 to 18 s. Retention time correction was achieved using the XCMS retcor function with the following parameter settings: minfrac=0.5,
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bw=10, mzwid=0.01. Missing peakdata were filled using the fillPeaks function. The CAMERA package was used to annotate isotope and adduct ions (http://bioconductor.org/packages/devel/bioc/html/CAMERA.html).

Inconsistent mass features were removed via the following steps. a) Delete all compounds with a retention time ≤ 40 sec, b) delete compounds within the last 30 sec of a measurement run, which are largely contaminants, c) delete compounds which the CAMERA package had identified as redundant isotopes ([M+1], [M+2], etc.), d) set peaks to 0, if relative intensity ≤ 90, and e) set all peaks of a mass feature within a treatment to 0 if their specific compound did not appear in ¾ of the replicates of one treatment. Finally, peak intensities of samples were normalized to the g fresh mass leaf material. The depurated peak intensity tables were converted into a comma-separated value-file format (*.csv) for upload to MetaboAnalyst (http://www.metaboanalyst.ca/) (Xia et al. 2009, 2012, Xia and Wishart 2011), a web server designed to facilitate metabolomics data processing and statistical analysis. The uploaded peak intensity table was processed without further data filtering, data normalisation or data transformation. The data was autoscaled to achieve normality and homoscedasticity and analysed by a principal component analysis (PCA) (see Fig. 9 and 10 for the PCAs).

2.8. Enzymatic quantification of starch and soluble sugars (glucose, fructose and sucrose) via spectrophotometric measurement

Soluble sugars were extracted from ~100 ± 5 mg leaf tissue per sample, ground with plastic pestles under liquid nitrogen, after the principle described in Machado et al. (2013). Per sample, soluble sugars were extracted three times from the leaf tissue; in a first step, 500 µL 80% (v/v) ethanol (denatured; Merck) were added, followed by incubation for 10 min at 78°C with constant shaking at 800 rpm (Thermomixer comfort; Eppendorf International) and 5 min centrifugation at 13,200 rpm at 4 °C (Microcentrifuge 5415R; Eppendorf International). In a second and third step, pellets were re-extracted twice with 500 µL 50% (v/v) ethanol, again followed by the incubation and centrifugation steps, as detailed above. For each sample, supernatants from all extraction steps were pooled in a well of a 96 deep-well plate (Nunc® 96 DeepWell™ plate, maximum volume 2 mL per well; Sigma-Aldrich) and stored at -
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20 °C until measurement. The remaining pellet was used for starch digestion and stored at -80 °C until enzymatic determination of starch content after the principle by Smith and Zeeman (2006).

Sugars can be quantified spectrophotometrically in a 96-well microplate after the general enzymatic principle for these assays as described in Velterop and Vos (2001).

![Figure 7: Principle of the enzymatic method used for spectrophotometric measurement of sugar content.](image)

Figure 7: Principle of the enzymatic method used for spectrophotometric measurement of sugar content. NADP$^+$ has an absorption peak at 260 nm. Since the produced NADPH has an additional absorption peak at 340 nm, it can be detected by measuring the extinction change at 340 nm in the course of the reaction (Munk et al. 2008a). G6P – glucose-6-phosphate; F6P – fructose-6-phosphate. NADP – nicotinamid-adenine-dinucleotide-phosphate. Picture was drawn by Ricardo Machado and is used in this thesis with his permission.

Preparation of enzymes and reactions was performed on ice. The master mix for the measurement was prepared with MilliQ water (Millipore, Bedford, MA, USA) and contained 50 mM Hepes buffer (pH 7.0)(Carl-Roth GmbH), 5 mM MgCl$_2$ (Sigma-Aldrich), 0.762 M NADP (NADP disodium salt; Calbiochem, Merck), 1.652 M ATP (adenosin-5’-triphosphat di-sodium salt, Carl-Roth GmbH) and 1 U/mL glucose-6-phosphat dehydrogenase (from yeast; Roche Diagnostics, Basel, Switzerland).

Enzymes were prepared in 50 mM HEPES buffer (pH 7.0, prepared with MilliQ water, 5 mM MgCl$_2$) as follows: For glucose measurement, 50 µL hexokinase (from yeast, 1500 U/mL; Roche Diagnostics) enzyme suspension were centrifuged and the pellet was re-suspended in 300 µL buffer, giving 250 U/mL. For fructose measurement, 50 µL phosphoglucoisomerase (from yeast, 10 mg in 1 mL, circa 350 U/mg; Roche Diagnostics) enzyme suspension were centrifuged and the pellet was re-suspended in 300 µL buffer, giving ca. 580 U/mL. For sucrose measurement, 50 mg invertase (from baker’s yeast, ≥ 300 U/mg solid; Sigma-Aldrich) were dissolved in 500 µL buffer, giving ≥ 30.000 U/mL. (One unit U describes enzyme...
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activity, equalling the amount of enzyme that converts 1 µmol substrate per minute, under defined optimized conditions (Munk et al. 2008b)).

For each sample or standard, 200 µL of master mix were dispensed per well of a 96 well microtiter plate (Greiner Bio-One International AG). The microplate contained a row of glucose standards (dilutions: 0 mM, 0.03125 mM, 0.0625 mM, 0.125 mM, 0.25 mM, 0.5 mM, 1 mM, 2 mM, 4 mM and 6 mM glucose) and technical replicates were not used. Depending on the expected sugar content, 25, 50 or 100 µL of each sample, and 25 or 50 µL of each glucose standards were pipetted to the respective wells on the microtiter plate. (Different volumes were noted on the pipetting scheme to calculate dilution factors accordingly.)

The completed microtiter plate was placed in the microplate reader Tecan infinite M200 (Tecan Group Ltd., Männedorf, Switzerland) and absorbance of formed NADPH at 340 nm was measured (against 405 nm as a blank), employing the software Tecan i-control (version 1.8, 2010, for infinite reader; Tecan Group Ltd.). In the automatic protocol employed, absorbance of all wells was measured every 1.5 minutes, for up to 30 cycles, with shaking between every measurement cycle to facilitate mixing-in of enzymes. The automatic protocol was manually terminated after absorbance readings at 340 nm had stabilized within the first two decimals. Between measurements of the individual sugars, the 96-well microtiter plate was removed from the microplate reader to add the next enzyme using a multipipette (Multipette Xstream, Eppendorf International) with a 100 µL tip (Combitips advanced, Eppendorf International). After initial stabilization of 340 nm absorbance readings, 2 µL hexokinase suspension (0.5 U) were added to each well for glucose determination. After subsequent stabilization of readings, 2 µL phosphoglucose isomerase suspension (1.2 U) were added to each well for fructose determination. Finally, following stabilization of readings, 4 µL invertase solution (≥ 120 U) were added to each well for sucrose determination.

Amounts of all three sugars are quantified as glucose equivalents, calculated via the glucose standard curve that correlated absorption increase at 340 nm with glucose standard concentration. Glucose equivalents were then converted to number of fructose and sucrose molecules in the sample; one measured glucose equivalent was equal to one molecule of the monosaccharide fructose in the sample, and for the
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disaccharide sucrose, two measured glucose equivalents were equal to one molecule sucrose in the sample (see Fig. 7)(Velterop and Vos 2001). Sugar content was calculated per g fresh mass of leaf material.

The remaining pellet was used for starch digestion and stored at -80 °C until enzymatic determination of starch content after the principle by Smith and Zeeman (2006). One mL of MilliQ water (Millipore, Bedford, MA, USA) was added per sample pellet. 100 µL of this suspension were pipetted into a screw cap vial and 500 µL of 1.22 mM fructose solution (Sigma-Aldrich) were added to each vial as a control. Similarly, 600 µL of different starch dilutions were prepared in screw cap vials (corn starch, Sigma-Aldrich; 0.1666, 0.833, 1.67, 2.5, 4 and 5 mg/mL) for a starch standard curve. Screw cap vials were closed tightly and autoclaved for 60 min at 120 °C in a plastic box wrapped in aluminium foil. For digestion, the following enzyme solution was prepared: Sodium acetate buffer (50 mM, pH 5.5; Sigma-Aldrich) with ca. 4 U/mL α-amyloglucosidase (from Aspergillus niger; Roche Diagnostics) and ca. 5.4 U/mL α-amylase (10 mg/mL; Roche Diagnostics); the enzyme solution was prepared with MilliQ water. After autoclaving, samples were allowed to cool and 500 µL enzyme solution were added to each vial, including the starch standards, and vials were incubated (4 hours, at 37 °C, at slow constant shaking). After incubation, solids of the suspension were left to precipitate at room temperature. Between starch digestion and measurement, vials were stored at –20°C. Spectrophotometric measurement of glucose from starch digestion and added fructose proceeded as detailed above for the soluble sugars glucose and fructose.

Fructose added to the starch pellet samples was measured as a quality control for pipetting, as there should be none in the starch standards. Absorption of the starch standards was used to calculate a standard curve of starch digestion that should at least partially have the shape of a saturation curve and is a test for homogenous autoclaving and starch digestion conditions across all screw vials. Starch content was calculated per g fresh mass of leaf material. From the measured glucose equivalents an approximation of the amount of starch in the sample can be calculated by multiplication of measured glucose equivalents with the molecular mass of anhydro-glucose (Smith and Zeeman 2006).
2.9. Temperature measurement in climate chambers

Infra-red radiation from the lamps in the climate chambers (spectrum in supplementary data, fig. S1) may have elevated temperatures above 24 °C during the light period. While a constant temperature was likely maintained under constant conditions (LL or DD), changes from light to dark under LD conditions might have caused fluctuations in air temperature.

In the caterpillar performance experiment, neonates, initially placed on the adaxial surface of rosette leaves, could be found all over the plant several days later, frequently sitting on the abaxial side or on the edge of leaves. Furthermore, observations of caterpillar behavior in the institute glasshouse, as well as in the field (e.g. Casey 1976), make it obvious that M. sexta larvae prefer to sit hidden under leaves. For these reasons, I employed temperature/light data loggers (HOBO UA-002-64 Pendant Temp/Light accuracy: temperature: ± 0.53 °C from 0 °C to 50 °C; logging interval: 10 min; logging time: at least 2 d; Onset Computer Corporation, Cape Cod, Massachusetts), placed shaded under rosette leaves of potted plants, at four positions in each of the two climate chambers. From the logged data, mean day and night temperatures were calculated for both climate chambers (see table S1 of the supplemental data).

2.10. Quantification of feeding activity

I used Vötsch growth chambers (VB 1014, Vötsch Industrietechnik GmbH, Balingen-Frommern, Germany) with the following settings, determined by temperatures measured as described above: for LL, 24 h light at 27 °C; for DD, 24 h dark at 24 °C; for LD, 12 h light at 26 °C, 12 h dark at 24 °C; humidity was set to 65%, the same as in the climate chambers, used for performance assays. Experiments began at 9 am, the same time used for the switch between light and dark periods in the climate chambers.

M. sexta eggs were incubated for 3 days in the in-phase climate chamber (see M. sexta performance experiments in 3.3) and after hatching, 9-12 larvae/plant were placed on 5 plants and reared for 3-4 days, until they were in the 2nd instar. The
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Rearing time was chosen such that majority of the caterpillars would not molt during 24 h of quantification in the respective growth chamber setting, as the development rate differed between growth chamber settings, probably due to temperature differences (see table S1 and S2 in the supplemental data).

Five larvae were placed in a square Petri dish (120 x 120 x 17 mm square polystyrene petri dish; Greiner Bio-One International AG, Kremsmünster, Austria) with plant material (n = 8 dishes) and acclimated under the light and temperature conditions of the respective assay (LL, DD or LD) for 24 h. Leaf material was provided as food during acclimation and exchanged every 12 h; the bases of leaves were covered with water-soaked tissues to prevent water loss. The food plants were cultivated under an in-phase light regime in one of the climate chambers. Immediately following the 24 h acclimation, the five larvae in each Petri dish were weighed together (2nd and early 3rd instar larvae; see Table S2 for the mass data; the initial larval mass at the start of a time-interval was used as an additional parameter in statistical analysis, detailed in section 2.11.) and provided with two fresh rosette leaves. After each 4 h interval (at 13:00, 17:00, 21:00, 1:00, 5:00 and 9:00), larvae were again weighed, old leaves were replaced with two freshly cut rosette leaves as described, and old leaves were scanned to quantify leaf area removed. For DD conditions and the dark phase of the diurnal conditions, the growth chamber was shielded from light with curtains; during work in the growth chamber, red light (λ = 720 nm) was used which is presumably invisible to M. sexta larvae. Furthermore, for the collection of rosette leaves in the dark phase of the climate chamber, green LED lights were used as previously described (see methods section 2.3.).

A 1 cm² piece of millimetric paper was scanned alongside leaves as a size standard. Lines were drawn with the line tool in Adobe Photoshop (version CS2; Adobe Systems GmbH, San Jose, CA, USA) if necessary to re-construct missing portions of the edges of leaves, and the wand tool was used if necessary to remove shadows originating from scanning; then missing leaf area was quantified with SigmaScan (SigmaScan Pro – Image Analysis, Version 5.0.0; Copyright 1987-1999 SPSS Inc.) in fill mode, with settings - fill: threshold method: auto threshold: % range: 25. Leaf area removed was normalized by dividing by the actual time between exchanges of
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leaves (3.7 to 4.3 h), and dividing by the mass of larvae in the Petri dish at the start of the respective time-interval. The feeding activity is presented as cm² g larval mass⁻¹ h⁻¹.

Out of the 40 larvae in each condition, only one died under LL, three under DD, and two under LD conditions (mortality: 2.5 to 7.5 %). Petri dish replicates with caterpillar mortality were excluded from data analysis, as were replicates in which larvae did not begin to feed within the first 4 h of the experiment, or in which the majority of larvae (i.e. 4 larvae per dish) began to molt; thus, the final number of replicates per assay was n = 5-6 (see also table S2 in the supplemental data).

2.11. Statistical analysis of data

Statistical tests of differences in M. sexta performance, targeted secondary metabolites, and sugar content were performed in R (versions 2.12.2 or 2.15.0) using RStudio (version 0.97.551)(Crawley 2013). Individual data sets, separated by time-points and treatments (in-phase and out-of-phase), were checked for normality – both graphically (quantile-quantile plots) and statistically (Shapiro-Wilk tests) – and F tests were used to check for homoscedasticity between the two treatments at individual time-points in the respective light regime. When the requirements of normality and homoscedasticity were met, Student’s t-tests were used to test for differences between in-phase and out-of-phase plants. When data failed to meet one requirement and could not be made to meet these requirements via log transformation, I conducted Wilcoxon rank-sum tests for independent samples.

Metabolomics data were analyzed in MetaboAnalyst (http://www.metaboanalyst.ca/; Xia et al. 2009, 2012, Xia and Wishart 2011). A principal component analysis (PCA) was conducted, after first autoscaling to achieve normality and homoscedasticity.

The data for the 24h quantification of feeding activity under diurnal conditions was analysed by the resident statistics expert of the Max Planck Institute for Chemical Ecology, Grit Kunert. Her work is credited in the paper following this diploma thesis (Herden et al., in prep.) and graphs with her statistical analysis are shown in this thesis with her permission (see section 3.5).
In Herden et al. (in prep.), the statistical method is described as follows: In order to investigate whether the leaf area consumed by *M. sexta* larvae is dependent on the time interval, and therefore probably also on the light and dark periods, linear mixed effects models (lme; nlme package; Pinheiro et al. 2013) were performed. Since initially heavier larvae consume more plant material, we corrected for this by using the consumed leaf area per mg larval mass as response variable (see supplementary Table S2). Explanatory variables were the time intervals (fixed effect), and the larva ID (random intercept). The optimal fixed structure was found by stepwise model simplification and factor level reduction followed by the comparison of the models with the likelihood ratio tests. This test was also used to obtain p-values. The minimal model was refitted with the restricted maximum likelihood method (REML) and validated for homogeneity of variances and normality of residuals (Zuur et al. 2009).

### 2.12. Preparation of figures

Figures were prepared with Microsoft PowerPoint 2010 (Microsoft Corporation), and Adobe Illustrator CS5 and Adobe Photoshop CS5 (Adobe Systems GmbH). The chemical structures in Fig. 6 were drawn with ChemBioDraw Ultra 13.0 (ChemBioOffice 2012; CambridgeSoft, Cambridge, Massachusetts, USA).
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3. Results

3.1. Performance of Manduca sexta larvae

Plants were entrained under one of two diurnal regimes: 12 h:12 h light:dark (in-phase) or 12 h:12 h dark:light (out-of-phase), and then infested with *M. sexta* larvae which had been entrained under the in-phase regime. As previously stated in the introduction, I started with the hypothesis that *M. sexta* larvae would perform better on out-of-phase plants than on in-phase plants, due to the presumed plant resistance-enhancing effect of synchronous entrainment on the plant.

For this reason, larval performance was measured as larval mass, from day 5 (approximately 2nd instar) until day 9 (4th, or 5th larval instar; the fifth is the final instar before pupation). The experiments had to be terminated at day 9, because larvae were now even capable of leaving their respective tray and feeding rates were increased in such a manner that one plant would not have lasted for three caterpillars for two days. In emulation of the experimental set-up of Goodspeed et al. (2012) and assuming potential negative effects of constant conditions on the induced defense response in *N. attenuata*, the experiment was repeated under three experimental conditions (Fig. 4): continuous light (LL), continuous dark (DD), and diurnal conditions (LD). At regular intervals, every 2-3 d, larvae were transferred to newly entrained plants.

Under all three conditions, growth of *M. sexta* larvae (Fig. 8) was similar on in-phase and out-of-phase plants (Wilcoxon rank sum tests for each day; **LL - day 5**: $n = 27-29$, $W = 412$, $P = 0.7368$, **day 7**: $n = 26-28$, $W = 286.5$, $P = 0.2511$, **day 9**: $n = 23-28$, $W = 300$, $P = 0.6866$; **DD – day 5**: $n = 35-37$, $W = 515.5$, $P = 0.1369$, **day 7**: $n = 31-36$, $W = 371$, $P = 0.0293$, **day 9**: $n = 30-35$, $W = 416$, $P = 0.1543$; **diurnal conditions – day 5**: $n = 35-44$, $W = 662$, $P = 0.2887$, **day 7**: $n = 34-41$, $W = 562$, $P = 0.1531$, **day 9**: $n = 26-41$, $W = 548$, $P = 0.8470$). There was only a single measurement, day 7 under DD, for which larvae feeding on out-of-phase plants were significantly heavier than larvae on in-phase plants ($P = 0.0293$). However, overall
growth rates of larvae were reduced under DD, as was mortality at the end of the 9 d experiment (mortality on in-phase and out-of-phase plant, under DD, 33.3 % and 20.0%, respectively; under LL, 48.9 % and 37.8%, respectively; under LD, 41.7% and 31.7%, respectively). While larvae on in-phase and out-of-phase plants developed through instars at similar rates, development was slower under DD conditions when compared to LL conditions (e.g. most frequent instar at day 9: DD, end of 3\textsuperscript{rd} to beginning of 4\textsuperscript{th} instar; LL, middle to end of 4\textsuperscript{th} instar).

Thus, a single measurement in DD is consistent with the hypothesis that in-phase plants are better defended. But overall, these results indicate that in-phase versus out-of-phase pairwise entrainment does not affect plant defense against \textit{M. sexta}. However, as already mentioned above, this effect was originally discovered in a plant-herbivore pair with a generalist herbivore (Goodspeed et al. 2012). The possibility was considered that \textit{M. sexta} larvae might not show differential development despite differences in the induced defense responses of the host plant due to its higher tolerance against certain defense strategies, e.g. nicotine (Wink and Theile 2002) and that, had a generalist been tested on \textit{N. attenuata}, one might have found differential performance. Therefore, in the next step, I tested for differences in the induced defense response of \textit{N. attenuata}, with untargeted and targeted methods.
Figure 8: *Manduca sexta* larvae performed similarly on in-phase and out-of-phase plants and *Nicotiana attenuata* plants were co-incubated under constant or diurnal conditions. *M. sexta* growth was monitored from day 5, to avoid damage to very young larvae, until day 9 (corresponding to the 2nd through 4th of 5 instars). Weighing of larvae coincided with the exchange of plants (see Fig. 4 for the experimental set-up). Wilcoxon rank sum tests were performed within each time point and light regime. The star * denotes a significant difference (P<0.05) between larvae on in-phase and out-of-phase plants on day 7 under DD conditions; in-phase and out-of-phase treatments did not differ significantly at any other time under any other conditions. Shown are means ± SE; number of larvae per treatment: for constant light (LL), n = 23-29; for constant dark (DD), n = 30-37; for diurnal conditions (LD), n = 35-46. Labels in LL apply to all charts.
3.2. Untargeted metabolite profiles of synchronous and asynchronous plants

Here, I compared the changes in leaves of similar rosette position before and after local damage by 3 days of *M. sexta* herbivory, in the first time-interval of the experiment of the respective light regime (Fig. 9). The degree of separation before and after herbivory is interpreted as strength of the induced defense response (Gaquerel et al. 2010).

Metabolomics profiles were similar for in-phase and out-of-phase *N. attenuata* rosette stage plants after 3 d of herbivory by *M. sexta* larvae under LL, DD, and LD. In principle components analyses (PCAs), 95% confidence intervals of metabolomic profiles of in-phase and out-of-phase plants did not overlap before herbivory in LL and LD, but clearly overlapped after 3 d of herbivory; similarly, overlap was greater in DD after 3 d of herbivory (Fig. 9).

The principal component axis 1 seems to mainly show the separation via the effects of herbivory and the principal component axis 2 seems to incorporate some components that might explain the separation between in-phase and out-of-phase treatment; effects of the respective light regime seem to affect separation along both axes. The separation of the four sample groups in Fig. 9 can be explained by the two principal components to 58% in LL, to 55.6% in DD, and 64.3% in diurnal conditions. However, the explanatory value of PCA axes for the separation of sample groups is difficult to compare between individual PCAs. Non-the-less, separation in metabolomics profiles before and after herbivory was more pronounced in LL and LD than in DD, indicating a stronger response of plants to herbivory in LL and LD.
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Figure 9: In-phase and out-of-phase Nicotiana attenuata plants respond similarly to Manduca sexta attack under constant or diurnal conditions. Principal component analyses (PCAs) were conducted on metabolomic profiles of plant samples (n = 5 plants/treatment) taken before or after M. sexta infestation for each regime. Dashed and dotted circles delineate 95% confidence intervals. Separation of 95% confidence intervals between sample groups stands for a statistically significant difference. Treatment groups were separated by the effects of continued exposure to the respective light regime (PC1 and PC2), induced defense in response to 3 d of herbivory (PC1) as well as effects due to prior entrainment conditions (PC2) and interactions of these factors.
Results

Figure 10: In-phase and out-of-phase *Nicotiana attenuata* plants respond similarly to *Manduca sexta* attack under constant or diurnal conditions. Principal component analyses (PCAs) were conducted on metabolomic profiles of plant samples (n = 5 plants/treatment) taken before and after 24h constant conditions pre-treatment, and after *M. sexta* infestation for each light regime. Drawn, dashed and dotted ovals delineate 95% confidence intervals. Separation of 95% confidence intervals between sample groups stands for a statistically significant difference. Treatment groups were separated by the effects of continued exposure to constant conditions (PC1 and PC2), induced defense in response to 3 d of herbivory (PC1), as well as effects due to prior entrainment conditions (PC2) and interactions of these factors.
Results

Metabolite profiles from in-phase and out-of-phase *Nicotiana attenuata* plants become less similar after 1 d exposure to constant light or constant dark, but more similar after 3 d of herbivory.

Additionally, for LL and DD conditions, changes brought about by the 24h pre-treatment in constant conditions are shown in Fig. 10, with data points for all three sampling time-points in constant conditions (see Fig. 4a for the experimental set-up). Transfer to constant conditions (LL and DD) itself had a strong effect on plant metabolite profiles: In PCAs for LL and DD that also incorporate data of treatment groups of samples taken before the 24h pre-treatment in constant conditions (see Figure 4a for the experimental set-up) show that in-phase and out-of-phase plants in fact became less similar after 24h exposure to constant conditions (Figure 10). The separation of the six treatment groups in Fig. 10 can be explained by the two principal components to 56.6% in LL and to 55.1% in DD.

A similar induced defense response in leaf tissue of in-phase and out-of-phase plants was also the overall trend in results of the quantification of target secondary metabolites.

3.3. Quantification of target secondary metabolites

We chose to quantify three metabolites from the wide range of secondary metabolites that *N. attenuata* accumulates in response to herbivory and for which an effect on *M. sexta* performance had been found in previous studies, nicotine, caffeoylputrescine (CP) and 17-hydroxygeranyllinalool-diterpene glycosides (HGL-DTGs)(Steppuhn et al. 2004, Heiling et al. 2010, Kaur et al. 2010). Furthermore, I quantified two metabolites in which usually no response to *M. sexta* herbivory is encountered: chlorogenic acid (CGA) and rutin (Kaur et al. 2010).

After quantification of secondary metabolite levels, Student’s t-tests were performed for normally distributed and homoscedastic data, and otherwise Wilcoxon rank sum tests were performed to compare independent samples between in-phase and out-of-phase treatments within sampling time-points of each light regime. (When the results
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of the tests are mentioned in the text, t-values and W-values make it apparent which of the two tests was used.)

3.3.1 Nicotine

Nicotine is a root-produced alkaloid in *N. attenuata* that can accumulate to concentrations up to 1% of dry mass in leaf material (Baldwin 1999). In the nicotine molecule two heterocyclic rings are joined together; the six-membered ring is derived from nicotinic acid and the five-membered ring is derived from the non-proteinogenic amino acid ornithine (Taiz and Zeiger 2006). The mechanism of biological activity in nicotine is via its interaction with acetylcholine receptors in the nervous system of animals (e.g. Schmeltz 1971, Govind et al. 2009). It is established that *M. sexta* larvae have a higher tolerance for nicotine than do generalist herbivores (Wink and Theile 2002), but that nicotine concentration can still impact larval performance (Steppuhn et al. 2004, Schuman and Baldwin 2012). Recent findings suggest that *M. sexta* larvae deal with toxic nicotine rather by rapid excretion than by oxidative detoxification (Kumar et al. 2014a, b).

As can be seen in figure 11, in-phase and out-of-phase plants under LL and LD had similar levels of nicotine before and after herbivory (Student’s t-test (*t*) or Wilcoxon rank sum test (*W*) for independent samples for each time-point; number of samples, *n* = 5 for all treatments; **LL**: 0 d - *t* = 0.4878, *df* = 8, *p*-value = 0.6388; 1 d - *t* = -2.1832, *df* = 8, *p*-value = 0.06056; 4 d - *t* = -0.2330, *df* = 8, *p*-value = 0.8216; **diurnal conditions**: 0 d - *t* = 1.5199, *df* = 8, *p*-value = 0.1670; 3 d - *t* = -1.491, *df* = 8, *p*-value = 0.1742). Reduced nicotine levels in out-of-phase plants, under DD, compared to in-phase plants might be an effect of the constant dark treatment, since the differences appeared before herbivory (Student’s t-test (*t*) or Wilcoxon rank sum test (*W*) for independent samples for each time-point; number of samples, *n* = 5 for all treatments **DD**: 0 d - *t* = 2.2878, *df* = 8, *p*-value = 0.05144; 1 d - *t* = 2.951, *df* = 8, *p*-value = 0.01840; 4 d - *t* = 2.901, *df* = 8, *p*-value = 0.0199). Induction of nicotine was not inhibited under any of the three different light regimes (Fig. 11). The increase of nicotine levels in DD and LL, after the 24h pre-treatment, but before herbivory (Fig. 11), is probably due to the mechanical damage inflicted by multiple samplings on the same plant, because nicotine also accumulates at higher levels,
systemically throughout the plant, after mere mechanical damage to plant tissue (Halitschke et al. 2000).

(See next page for figure caption.)
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Figure 11: Nicotine accumulation in *N. attenuata* rosette-stage plants after 3 days *M. sexta* herbivory under different light regimes. Plant material was collected at three time-points under DD and LL (0 d in continuous conditions, 1 d in continuous conditions, and 4 d in continuous conditions [= 3 d herbivory under continuous conditions]) and two time-points under diurnal conditions (LD) (0 d of herbivory, and 3 d of herbivory). Nicotine concentrations between in-phase and out-of-phase plants of a time-point were compared with either student t-tests or Wilcoxon rank sum tests for independent samples (see text for the respective details). Number of samples, n = 5 for all treatments. Stars denote significant differences between in-phase and out-of-phase plants (* - p-value < 0.05, ** - p-value < 0.01, *** - p-value < 0.001).

### 3.3.2. Phenolics

Phenolics are characterized by the fact that they contain a phenol group (a benzene ring with a hydroxyl functional group) and are mainly synthesized by the plastidial shikimic acid pathway from the precursor phenylalanine. Phenylpropanoids, defined by a phenol group and an attached three-carbon chain, are the first products of phenolic biosynthesis and these simple phenolics are themselves precursor for the biosynthesis of more complex phenolics; phenylpropanoids are also needed for lignin biosynthesis (Taiz and Zeiger 2006). All phenolic compounds tested here (CP, CGA and rutin) incorporate phenylpropanoid structures in their biosynthesis (Kaur et al. 2010).

In addition to caffeoylputrescine, I also quantified the chlorogenic acid (CGA) and the rutin content of attacked plant tissues, because these metabolites are usually not induced upon *M. sexta* herbivory (Keinänen et al. 2001, Kaur et al. 2010) and might reflect the impact of abiotic stressors under constant conditions on plant physiology. (Brouquisse et al. 1998, Graf et al. 2010, Sysoeva et al. 2010).

#### 3.3.2.1. Caffeoylputrescine

Caffeoylputrescine is a phenylpropanoid-polyamine conjugate (Kaur et al. 2010, Bassard et al. 2010, Onkokesung et al. 2012). *N. attenuata* plant leaf tissue contains low constitutive amounts of caffeoylputrescine, but after *M. sexta* herbivory accumulates the compound to several times the constitutive concentrations (Kaur et al. 2010). Furthermore, when *M. sexta* neonates were fed on leaves of *N. attenuata* plants, silenced in CP biosynthesis, and, in one treatment, leaves were sprayed with caffeoylputrescine, *M. sexta* larvae were significantly lighter and thus, showed a
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Reduced performance relative to controls (Kaur et al. 2010). However, the mechanism of biological activity for CP is to date unknown (Kaur et al. 2010, Onkokesung et al. 2012).

As is evident from figure 12, caffeoylputrescine was only found in quantifiable levels after 3 days of *M. sexta* herbivory, and then, only in LL and LD. This means that CP biosynthesis and accumulation was probably inhibited under DD and this metabolite likely had no influence on *M. sexta* performance in DD. For LL, contrary to expectations, out-of-phase plants contained significantly more CP than in-phase plants (LL: 4 d - n = 5 for both treatments, student’s t-test, t = -2.649, df = 8, p-value = 0.0293). Under diurnal conditions, however, there were no significant difference between treatments after 3 days *M. sexta* herbivory (LD: 4 d – n = 5 for both treatments, student’s t-test, t = -0.8567, df = 8, p-value = 0.4165).

**Figure 12:** Caffeoylputrescine accumulation in *N. attenuata* rosette-stage plants after 3 days *M. sexta* herbivory under different light regimes. Since the compound could only be detected at quantifiable levels after 3 days herbivory, the other time-points are not shown. Plots show the situation for 4 d of constant conditions (LL and DD) and 3 d herbivory in diurnal conditions (LD), respectively. CP concentrations between in-phase and out-of-phase plants of a time-point were compared with student t-tests for independent samples (see text for the respective details). Number of samples, n = 5 for all treatments. Stars denote significant differences between in-phase and out-of-phase plants (* - p-value < 0.05, ** - p-value < 0.01, *** - p-value < 0.001).
3.3.2.2. Chlorogenic acid

Chlorogenic acid (CGA) is found in two isomers in *N. attenuata* plant tissue: 4- and 5-caffeoylquinic acid which are called cryptochlorogenic acid and chlorogenic acid, respectively (Keinänen et al. 2001). For quantification, peak areas of both isomers were combined to give the total CGA content (Fig. 13); of the two isomers cryptochlorogenic acid accumulated to lower levels than chlorogenic acid (data not shown). Some studies have reported increased CGA levels after simulated herbivory, while others have found no increase (Keinänen et al. 2001). There is also some evidence that CGA responds to UV-B exposure (Izaguirre et al. 2007). As can be seen in figure 13, despite the supposedly “neutral role” of CGA, total CGA levels had dropped after herbivory in constant light and, similarly, in LD. On the other hand, that total CGA levels fell the longer plants were exposed to DD (Fig. 13), which might be an indicator of degradation processes in plant tissues (Baysdorfer et al. 1988, Brouquisse et al. 1998, Gibon et al. 2004). Out-of-phase plants contained significantly more total CGA than in-phase plants under LL at 0 d and at 4 d in constant conditions (Student’s t-test (t) for independent samples for each time-point; number of samples, n = 5 for all treatments; LL: 0 d - t = -3.2225, df = 8, p-value = 0.01219; 1 d - t = -2.0308, df = 8, p-value = 0.07676; 4 d - t = -2.9841, df = 8, p-value = 0.01749). Similarly, out-of-phase plants contained more total CGA than in-phase plants in diurnal conditions at 0 d of herbivory (Wilcoxon rank sum test (W) for independent samples for each time-point; number of samples, n = 5 for all treatments; LD: 0 d - W = 0, p-value = 0.007937; 3 d - W = 3, p-value = 0.05556). These differences might either point to slight differences in physiology between treatments due to different entrainment regimes, or even more likely might be due to the low statistical power of the small sample size of only 5 biological replicates per treatment which makes these statistical differences less believable (Button et al. 2013). Falling total CGA levels in DD (Fig. 13) might be a consequence of exposure to constant darkness, since the reduction is already noticeable after 1 d under constant conditions. No other tendencies were observed under DD (Student’s t-test (t) for independent samples for each time-point; number of samples, n = 5 for all treatments; DD: 0 d - t = -1.5893, df = 8, p-value = 0.1507; 1 d - t = -0.9514, df = 8, p-value = 0.3692; 4 d - t = -1.6646, df = 8, p-value = 0.1345).
Results

(See next page for figure caption.)
Results

Figure 13: Accumulation of total CGA in *N. attenuata* rosette-stage plants after 3 days *M. sexta* herbivory under different light regimes. Plant material was collected at three time-points under DD and LL (0 d in continuous conditions, 1 d in continuous conditions, and 4 d in continuous conditions (3 d herbivory under continuous conditions)) and two time-points under diurnal conditions (LD) (0 d of herbivory, and 3 d of herbivory). Total CGA concentrations between in-phase and out-of-phase plants of a time-point were compared with either student t-tests or Wilcoxon rank sum tests for independent samples (see text for the respective details). Number of samples, n = 5 for all treatments. Stars denote significant differences between in-phase and out-of-phase plants (* - p-value < 0.05, ** - p-value < 0.01, *** - p-value < 0.001).

3.3.2.3. Rutin

Rutin does not respond to herbivory, but increases strongly under UV-B exposure (e.g. Izaguirre et al. 2007, Dinh et al. 2013), indicating a potential role in protection against high irradiance levels under natural conditions.

As can be seen in figure 14, levels of rutin in LD remained unaffected by entrainment regime or herbivory (Student’s t-test (t) or Wilcoxon rank sum test (W) for independent samples for each time-point; number of samples, n = 5 for all treatments; LD: 0 d - W = 4, p-value = 0.09524; 3 d - t = -0.2386, df = 8, p-value = 0.8174). But as previously seen in CGA levels, under DD, out-of-phase plants contained more rutin than in-phase, at 0 d and at 1 d in constant conditions (Fig. 14); probably again due to effects caused by the small number of biological replicates (Button et al. 2013)(Student’s t-test (t) or Wilcoxon rank sum test (W) for independent samples for each time-point; number of samples, n = 5 for all treatments; DD: 0 d - t = -2.7617, df = 8, p-value = 0.02461; 1 d - W = 0, p-value = 0.007937; 4 d - t = -1.8435, df = 8, p-value = 0.1025). Also, under DD, rutin levels fall the longer plants are exposed to constant conditions, maybe indicating physiological stress or degradation of compounds (Brouquisse et al. 1998, Gibon et al. 2004, Graf et al. 2010). Under LL, out-of-phase plants contained significantly more rutin than in-phase plants at 1 d in constant conditions (Fig. 14), but this is probably again due to effects of small sample size (Button et al. 2013)(Student’s t-test (t) or Wilcoxon rank sum test (W) for independent samples for each time-point; number of samples, n = 5 for all treatments, LL: 0 d - W = 9, p-value = 0.5476; 1 d - t = -3.2016, df = 8, p-value = 0.01258; 4 d - t = -0.3494, df = 8, p-value = 0.7358). Furthermore, rutin levels were reduced in LL after herbivory, in both in-phase and out-of-phase plants, at 4 d in constant conditions, due to unknown reasons.
Results

(See next page for figure caption.)
**Figure 14:** Accumulation of rutin in *N. attenuata* rosette stage plants after 3 days *M. sexta* herbivory under different light regimes. Plant material was collected at three time-points under DD and LL (0 d in continuous conditions, 1 d in continuous conditions, and 4 d in continuous conditions [= 3 d herbivory under continuous conditions]) and two time-points under diurnal conditions (LD) (0 d of herbivory, and 3 d of herbivory). Rutin concentrations between in-phase and out-of-phase plants of a time-point were compared with either student’s t-tests or Wilcoxon rank sum tests for independent samples (see text for the respective details). Number of samples, n = 5 for all treatments. Stars denote significant differences between in-phase and out-of-phase plants (* - p-value < 0.05, ** - p-value < 0.01, *** - p-value < 0.001).

### 3.3.3. 17-hydroxygeranyllinalool-diterpene glycosides (HGL-DTGs)

17-hydroxygeranyllinalool diterpene glycosides (HGL-DTGs) are a class of terpenoid secondary metabolites occurring in the aboveground tissue of *N. attenuata*, comprising 11 different compounds (the simplest, lyciumoside I, is depicted in **Fig. 6 in the methods section**). HGL-DTGs have an acyclic C$_{20}$ diterpene backbone to which sugar groups (glucose and rhamnose) are conjugated; these sugar groups in turn have malonyl groups conjugated to them. To date the specific mechanism of their action on *M. sexta* is not known, although reduced total HGL-DTG accumulation significantly increased *M. sexta* performance. The diterpene skeleton of HGL-DTGs in *N. attenuata* shoot tissue is synthesized via the plastidial route of terpene synthesis, since reduced expression of an isoform of a key enzyme of this pathway reduced HGL-DTG accumulation (Heiling et al. 2010).

As in the other two defense compounds, nicotine and CP, there were no consistent differences in HGL-DTG content between in-phase and out-phase plants (**Fig. 15**). Before herbivory, plants in LD accumulated HGL-DTGs in unquantifiable or only low constitutive amounts (average in out-of-phase plants: 9.182 ± 0.803 (± SE) relative peak area/g fresh mass) and the levels of induced HGL-DTGs are not affected by the presumed re-entrainment process in out-of-phase plants, since, after 3 d of herbivory, plants of both treatments had accumulated similar levels of HGL-DTGs (LD: 3 d – n = 5 for both treatments, student’s t-test, t = 0.9371, df = 8, p-value = 0.3761).
Results

(See next page for figure caption.)
Figure 15: Accumulation of HGL-DTGs in *N. attenuata* rosette stage plants after 3 days *M. sexta* herbivory under different light regimes. Plant material was collected at three time-points under DD and LL (0 d in continuous conditions, 1 d in continuous conditions, and 4 d in continuous conditions [= 3 d herbivory under continuous conditions]) and two time-points under diurnal conditions (LD) (0 d of herbivory, and 3 d of herbivory). Relative contents of HGL-DTGs were compared between in-phase and out-of-phase plants of a time-point by either student t-tests or Wilcoxon rank sum tests for independent samples (see text for the respective details). Number of samples, n = 5 for all treatments. Stars denote significant differences between in-phase and out-of-phase plants (* - p-value < 0.05, ** - p-value < 0.01, *** - p-value < 0.001).

Differences between in-phase and out-of-phase plants under LL (Student’s t-test (t) for independent samples for each time-point; number of samples, n = 5 for all treatments; LL: 0 d - t = 0.1363, df = 8, p-value = 0.895; 1 d - t = -4.73, df = 8, p-value = 0.001483; 4 d - t = 1.428, df = 8, p-value = 0.1912) or DD (Student’s t-test (t) for independent samples for each time-point; number of samples, n = 5 for all treatments; DD: 0 d - t = 2.8407, df = 8, p-value = 0.02179; 1 d - t = 2.0282, df = 8, p-value = 0.07708; 4 d - t = 2.327, df = 8, p-value = 0.0484) might again be due to effects of the small sample size on statistics (Button et al. 2013) or different capability to deal with stress under constant light conditions (Sysoeva et al. 2010). When compared to the induced defense response under diurnal conditions, prominent tendencies in HGL-DTG concentrations are the strong induction of HGL-DTG levels under constant light conditions and the failure of plants under DD to show an induced accumulation in HGL-DTGs (Fig. 15). This could hint at a connection between light availability, photosynthesis and HGL-DTG biosynthesis, as the diterpene backbone is synthesized by the plastidial route of terpene synthesis; furthermore, the negative connection between in DD and HGL-DTG content might indicate that, under carbon starvation (section 3.4.), the needs for sugar moieties for HGL-DTG biosynthesis (Heiling et al. 2010) are not met.

Overall, these observations for five different metabolites (Fig. 11, 12, 13, 14, and 15) are consistent with the changes in general metabolite profiles after herbivory (Fig. 9 and 10), indicating that a similar induced defense response takes place in in-phase and out-of-phase plants. For the expression of the induced defense response, proper entrainment with the environment does not seem to play a large role, but different light regimes can constrain this induced response as seen in the failure of plants to accumulate CP and HGL-DTGs after 3 d herbivory in DD (Fig. 12 and 15).
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Falling levels in rutin and total CGA in DD could hint at a degradation of compounds within the leaves during a starvation response (Baysdorfer et al. 1988, Brouquisse et al. 1998, Gibon et al. 2004, Graf et al. 2010), as data in the following section 3.4. support that carbon starvation takes place in *N. attenuata* in DD. Furthermore, constant light might lead to stronger accumulation of HGL-DTGs due to increased availability of energy and precursors to the plant. The induced nicotine response, on the other hand, seems to be quite robust to changes in light availability.

Small statistically significant differences in compound levels appear sporadically at several time-points (Fig. 13, 14 and 15). For example, there are both a case in total CGA and rutin with differences between out-of-phase plants and in-phase plants at the start of the experiment, before any experimental influence (Fig. 13 and 14), that do not appear in the other plants sampled at the same time-point for another light regime. This is surprising, since plants for the LL and DD experiments originated from the same in-phase and out-of-phase entrained plant batches. Any small significant difference could be a product of the low power of the small sample size of biological replicates in each treatment (Button et al. 2013), making small differences between treatments after 3 d herbivory less plausible. On the other hand, there are some general trends which both in-phase and out-of-phase plants follow and which, thus, are more believable.

3.4. Quantification of starch and soluble sugars (glucose, fructose and sucrose)

Induced responses to herbivory comprise changes in secondary and primary metabolism (Schwachtje et al. 2006, Schwachtje and Baldwin 2008). In addition to target secondary metabolites, starch and soluble sugar levels (glucose, fructose and sucrose) were quantified (as detailed in section 2.8.; see Fig. 16, S2, S3 and S4). However, data from soluble sugar levels is rather preliminary and incomplete, and, additionally, is difficult to interpret because e.g. levels of glucose might be relatively responsive to photosynthetic activity, as I saw in a preliminary experiment in which belated sampling after lights-on led to similar glucose levels in in-phase and out-of-phase plants although in-phase plants had only been exposed to a few minutes of light (data not shown). Therefore, quantified soluble sugars levels at least for the
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Monosaccharides glucose and fructose might be transient. On the other hand, sucrose and starch levels usually reflect the partitioning of the plant between immediate demands for carbon (e.g. for growth) and anticipated demands (e.g. for maintained processes during the night), as sucrose is transported via the phloem to designated sites and transitory starch is stored in chloroplasts of the leaves (Smith and Stitt 2007). Hence, sucrose and starch levels might be better indicators of present carbon reserves and allocation.

After quantification of starch and soluble sugar levels, Student’s t-tests or Wilcoxon rank sum tests, in case of missing normality or homoscedasticity, were performed to compare independent samples between in-phase and out-of-phase treatments within sampling time-points of each light regime. (When the results of the tests are mentioned in the text, t-values and W-values make it apparent which of the two tests was used.) Due to the preliminary quality of the data for the soluble sugars, quantification or statistical tests were not possible at a number of time-points, especially in samples from the LD setting.

Carbon reserves in samples from in-phase plants, collected before 24h pre-treatment in constant conditions or before herbivory in diurnal conditions, should present the state at the end of a 12h night (see Fig. 4 for the experimental set-up), while samples collected at the same time-point in out-of-phase plants should present the state in carbon reserves at the end of a 12h day. In accordance with this fact, starch levels were significantly different in in-phase and out-of-phase plants at the first sampling time-point in all three light settings (Student’s t-test (t) or Wilcoxon rank sum test (W) for independent samples for each time-point; LL: at 0 days - n = 5 in both treatments, W = 0, p-value = 0.007937; DD: at 0 days - n = 5 in both treatments, t = -16.843, df = 8, p-value = 1.564e-07; LD: at 0 days - n = 7 in both treatments, W = 0, p-value = 0.0005828).
(See next page for figure caption.)
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**Figure 16:** Accumulation of starch in leaves of *N. attenuata* rosette stage plants after 3 days *M. sexta* herbivory under different light regimes. Plant material was collected at three time-points under DD and LL (0 d in continuous conditions, 1 d in continuous conditions, and 4 d in continuous conditions [= 3 d herbivory under continuous conditions]) and two time-points under diurnal conditions (LD) (0 d of herbivory, and 3 d of herbivory). Relative contents of starch were compared between in-phase and out-of-phase plants of a time-point by either student t-tests or Wilcoxon rank sum tests for independent samples (see text for the respective details). Number of samples, n = 5 for constant conditions, and n = 7-8 for diurnal conditions; but see text for details. Stars denote significant differences between in-phase and out-of-phase plants (* - p-value < 0.05, ** - p-value < 0.01, *** - p-value < 0.001).

When samples were collected after 24h pre-treatment in constant conditions, starch levels in in-phase and out-of-phase plants in LL were even higher than normally encountered in plants at the end of a 12h night (compare with starch levels in out-of-phase plants of the first sampling time-point; Fig. 16), however, the significant difference previously seen at the first sampling time-point was retained and out-of-phase plants accumulated higher levels of starch than in-phase plants (**LL: at 1 days - n = 5 in both treatments, two sample t-test, t = -3.8544, df = 8, p-value = 0.004847**). In contrast, after 24h pre-treatment in constant dark, starch levels in in-phase and out-of-phase plants in DD were similar (**DD: at 1 days - n = 5 in both treatments, two sample t-test, t = 0.5037, df = 8, p-value = 0.6281**) and were even slightly lower than in in-phase plants at the end of a 12h night (Fig. 16).

After 3 days herbivory and 4 days in constant conditions, starch levels in-phase and out-of-phase plants under LL were again at normal physiological levels and had become similar (**LL: at 4 days – in-phase n = 4, out-of-phase n = 5, two sample t-test, t = 1.6093, df = 7, p-value = 0.1516**). However, the normalizing agent is unclear. On the other hand, at the third sampling time-point starch levels in in-phase and out-of-phase plants in DD had not fallen further (**DD – at 4 days - n = 5 in both treatments, two sample t-test, t = -1.5183, df = 8, p-value = 0.1674**). In diurnal conditions, starch levels in in-phase and out-of-phase plants were similar after 3 days of herbivory (**LD: at 3 days - n = 8 in both treatments, two sample t-test, t = 1.2069, df = 14, p-value = 0.2475**), maybe indicating that out-of-phase plants had already recovered their carbon balance.
Overall, soluble sugar levels for LL conditions followed the pattern described for starch levels, with sucrose levels slightly elevated above normal physiological levels in out-of-phase levels, after 24h in constant light (Fig. S4). But, in the case of glucose and fructose (Fig. S2 and S3), levels were elevated above normal physiological levels in in-phase plants rather than out-of-phase plants, after exposure to 24h of constant light. As stated previously, because of the transient character of glucose, monosaccharide levels and, hence, these different patterns are difficult to interpret. Some studies have observed a down-regulation of photosynthesis rate under constant light (van Gestel et al. 2005, Sysoeva et al. 2010), and maybe the reduction of starch levels in LL after 3 d herbivory is related to this. Apart from regulation of carbon assimilation, export and storage after continued exposure to constant light, it cannot be decided clearly whether the normalization of starch and soluble sugar levels to normal physiological levels after 3 days of herbivory and 4 days in constant light might not be simple due to an induced response in which export of carbon from the damage tissue increases (Schwachtje et al. 2006).

In the end, in constant light, statistical analysis of differences between in-phase and out-of-phase plants in soluble sugar levels showed a similar pattern as in starch levels, statistically significant differences before and after 24 pre-treatment in constant light and similar levels after 3 days herbivory in constant light, for glucose (Student’s t-test for each time-point; n = 5 for all treatments; **LL:** 0 days - t = -2.6433, df = 8, p-value = 0.02956; 1 days - t = 6.156, df = 8, p-value = 0.0002722; 4 days - t = 0.8656, df = 8, p-value = 0.4119), for fructose (Student’s t-test for each time-point; **LL:** 0 days – in-phase n = 4, out-of-phase n = 5, t = -7.8335, df = 7, p-value = 0.0001042; 1 days - n = 5 in both treatments, t = 3.5174, df = 8, p-value = 0.007875; 4 days - n = 5 in both treatments, t = 0.4474, df = 8, p-value = 0.6665) and sucrose (Student’s t-test for each time-point; n = 5 for all treatments; **LL:** 0 days - t = -11.9403, df = 8, p-value = 2.227e-06; 1 days - t = -5.9242, df = 8, p-value = 0.0003520; 4 days - t = 0.0975, df = 8, p-value = 0.9247).

In contrast to the rather complex changes in carbohydrate dynamics taking place under constant light conditions, changes in starch and soluble sugar levels in DD, after exposure to 24h pre-treatment and subsequent 3 days herbivory in constant darkness, can be described with the term “carbon depletion”. After 24h in constant
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dark, levels in starch and all three soluble sugars (Fig. 16, S2, S3, and S4) were depleted below levels encountered at the end of a 12h night, for both in-phase and out-of-phase plants. After 3 days of herbivory in constant darkness, starch and soluble sugar levels in in-phase and out-of-phase plants had not fallen further, which might indicate that minimum levels had already been reached after 24h in DD. This would be in line with studies that have shown that *A. thaliana* plants exhaust their starch reserves by the time of the next anticipated dawn, with the anticipation process being under the control of the circadian clock (Graf et al. 2010). Regarding the rapid depletion in levels of soluble sugars and starch, it is not surprising that differences between in-phase and out-of-phase plants, as they existed before transfer to constant darkness, rapidly disappeared. However, small significant differences could be recovered that, again, like in the quantification of target secondary metabolites, might be attributable to the small sample sizes, used for the statistical tests (Button et al. 2013); for glucose (Student’s t-test (t) or Wilcoxon rank sum test (W) for independent samples for each time-point; **DD: 0 days** - n = 5 in both treatments, t = -8.7045, df = 8, p-value = 2.367e-05; **1 days** – not testable, in-phase n = 4, out-of-phase n = 3; **4 days** - inphase n = 4, outofphase n = 5, W = 0, p-value = 0.01587), for fructose (Student’s t-test (t) for each time-point; **DD: 0 days** - n = 5 in both treatments, t = -10.3984, df = 8, p-value = 6.336e-06; **1 days** - n = 5 in both treatments, t = 1.2045, df = 8, p-value = 0.2628; **4 days** – not testable, in-phase n = 5, out-of-phase n = 3) and, for sucrose (Student’s t-test (t) or Wilcoxon rank sum test (W) for independent samples for each time-point; **DD: 0 days** - n = 5 in both treatments, t = -7.6841, df = 8, p-value = 5.829e-05; **1 days** - n = 5 in both treatments, W = 19, p-value = 0.2222; **4 days** – not testable, in-phase n = 3, out-of-phase n = 5).

As has been shown in previous studies, in response to changing day and night length, plants adjust the partitioning of their photosynthetic assimilate to starch synthesis, but also adjust starch degradation rate at night (Smith and Stitt 2007, Sulpice et al. 2014). The regulation in response to changing day length is still mostly unclear (Smith and Stitt 2007), although the circadian clock controls the rate of starch degradation at night (Graf et al. 2010). However, it is unclear whether similar processes were involved in the adjustment of the carbon dynamics in “jet-lagged”
out-of-phase to the new 12h: 12h light regime, 12h off from the previous entrainment regime.

As is obvious from the experimental design of the LD experiment (Fig. 4), out-of-phase plants experienced 12h of light before transfer to the new light regime, at lights-on. If out-of-phase plants should have reacted similar to an extended day, as did plants in constant light (Fig. 16), it is likely that these plants accumulated excess carbon reserves, above levels at the end of a 12h day, and thus might have dealt better with the first unexpected night, 12h after transfer.

However out-of-phase plants dealt with the need to re-entrain to the new light regime, after 3 days of herbivory in the new light regime, leaves on in-phase and out-of-phase plants contained similar levels of starch at the end of a 12h night, perhaps implying that carbon dynamics were already adjusted in out-of-phase plants. But, the extent to which 3 days herbivory had an effect on the adjustment of carbon dynamics in out-of-phase plants, cannot be said.

In agreement to similar carbon dynamics in in-phase and out-of-phase plants after 3 days herbivory, soluble sugar levels were not significantly different at the second sampling time-point, for those preliminary data that could be tested; for glucose (LD: 0 days – not testable, because data from in-phase was not quantifiable; 3 days – not testable, in-phase n = 4, out-of-phase n = 3), for fructose (LD: 0 days – not testable, because data from in-phase was not quantifiable; 3 days - n = 5 in both treatments, two sample t-test, t = -0.396, df = 8, p-value = 0.7024) and, for sucrose (LD: 0 days - n = 7 in both treatments, two sample t-test, t = -8.3443, df = 12, p-value = 2.436e-06; 3 days - not testable, because data was not quantifiable).

Overall, levels of starch and soluble sugars under LL and LD are difficult to interpret, but might indicate down-regulation of photosynthesis rates under constant light in plants of both treatments, and a rapid re-adjustment of carbon dynamics in “jet-lagged” out-of-phase plants, respectively. In DD, rapidly plummeting levels of starch and soluble sugars show the effects of carbon starvation.
3.5. Changes in plant phenotypes after exposure to constant conditions

Despite reservations voiced previously, plants did not show obvious signs of photodamage (e.g. chlorosis or necrotic tissue; Sysoeva et al. 2010) under constant light conditions, however, exposure was only for 4 days and might have been too short for effects of this kind (see Fig. 17A for phenotypes after different numbers of days in LL). Furthermore, after return to diurnal conditions, plants with LL treatment showed growth similar to plants without exposure to constant light or were even further elongated (see Fig. S5 in the supplementary data).

Figure 17: Phenotypes of plants after exposure to constant conditions. While exposure to constant light (LL) produced no discernible changes in plant phenotype, exposure to constant darkness (DD) produced a change in phenotype that is reminiscent of shade avoidance syndrome. Photos were taken by Jasmin Herden.

In contrast to this, plants exposed to DD started to show changes in phenotype within 24h of constant darkness: The first changes observed were an upward movement of rosette leaves (see Fig. 17B for phenotypes after different numbers of days in DD). Plants that were exposed to up to 4 days additionally showed etiolated younger rosette leaves and the leaves seemed to have lost turgidity and were “floppy” (personal observation). But these rather drastic changes in phenotype were reversible after transfer back to diurnal conditions, and plants only showed a slight delay in elongation (see Fig. S5 in the supplementary data; personal observation). The upward movement of leaves is reminiscent of symptoms usually encountered during
shading and described as part of the shade avoidance syndrome (SAS) (Pierik et al. 2004, Franklin 2008, Keuskamp et al. 2010, 2012); in this context this reversible change in leaf movement is called hyponasty.

3.6. Quantification of feeding activity in Manduca sexta larvae under diurnal and constant conditions

As stated previously (see methods section 2.9), temperatures were measured in climate chambers shaded under leaves, employing data loggers. Indeed, despite the setting of the climate chambers to 24 °C, slight temperature differences were measured between the 12h lights-on and 12h lights-off period, even with slightly different average day temperatures between both climate chambers (see table S1 in the supplemental data). These temperature differences between photophase and scotophase might be the product of the lamps in the climate chamber that also had emittance peaks in the infrared range (see Fig. S1 in the supplemental data for a representative spectrum of the lamps used). Temperature is an important determinant for *M. sexta* feeding activity (Kingsolver and Woods 1997a, Petersen et al. 2000) and diurnal conditions with fluctuating temperatures between day and night might bring about fluctuations in feeding activity.

The quantification of consumed leaf area was measured under LL, DD and LD conditions; the Vötsch growth chambers used for incubation were set to the average day temperature of climate chambers for LL, to the average night temperature of climate chambers for DD, and to a 12h:12h alternation of the average day and night temperatures of the climate chamber, used for the LD caterpillar performance assay, for LD (see table S1 in the supplementary data). Hence, *M. sexta* larval feeding activity, in terms of leaf area removed per time-interval, was quantified under the following incubation temperature and light regimes: LL at 27 °C, DD at 24 °C, and LD with a changing regime of 12 h L (26 °C):12 h D (24 °C). Quantification was preceded by 24h acclimatization to the incubation conditions in the growth chambers; larvae were 3 to 4 days post-hatching at the start of the acclimatization period, and ages were selected so that the majority of the larvae would be feeding and be between molting events during the 24h following the acclimatization period.
Larvae were fed on freshly cut *N. attenuata* rosette leaves from a common pool of plants under in-phase LD conditions, and leaves were exchanged at 4h intervals. Finally, leaf area removed, quantified in cm$^2$, was normalized by the actual time between exchange of leaves (3.8 to 4.3 hours) and cumulative larval mass in the respective square Petri dish at the start of the respective time-interval.

Damage rates increased slightly over the 24 h of the experiment, under LL, with a slight reduction toward the end of the experiment, which can likely be attributed to initiation of molting by larvae (Figure 18A). Similarly, there was an increase in damage rate over the course of the experiment, under DD (Figure 18B). Under both LL and DD, there were no discernible 24h fluctuations in feeding activity (Figure 18A and B). Since damage in leaf area removed was normalized by the initial larval mass which, of course, increased as larvae grew (see table S2) the actual amount of leaf area removed increased during the 24h, and the low slope of the increase in damage rates under the constant conditions rather highlights the strong interdependence of larval size and the damage, it can deal out.

Under LD conditions, larval feeding activity increased over the first three time-intervals, but dropped in the 4th time-interval, which followed the switch to the night regime (Figure 18C). However, in the 5th and 6th time-interval, in the middle and end of the night period, larval feeding activity increased again. This fluctuation in feeding activity under diurnal conditions was subjected to a statistical analysis by the institute’s resident statistics specialist, Grit Kunert (see methods section 2.11.).

The results of the statistical analysis, that compared feeding activity in all six time-intervals and took larval mass at the start of the respective time-interval into account as a covariable, showed that damage rates measured in the first time-interval, from 9:00 to 13:00, and in the 4th, 5th and 6th time-intervals from 21:00 to 09:00 were significantly lower than rates measured in the 2nd and 3rd time-interval between 13:00 and 21:00, when accounting for initial larval mass in a minimal linear mixed effects model, $t = 6.274$, df = 29, $P < 0.0001$ (see methods section 2.11. for details of the statistical procedure). Fluctuations between the damage rates at day and night could possibly be due to altered light conditions or incubation temperatures between day and night. Similarly, differences in the magnitude of damage rates between the three
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settings (i.e. damage in terms of leaf area removed was highest in the LL setting and was lowest in the DD setting; see different scales of the figures), as they are discussed here, are probably due to a combination of slightly different larval age, and different incubation temperature, as increased temperatures elevate feeding rates (Kingsolver and Woods 1997a) and, consequently, increase developmental rates of *M. sexta* larvae (Kingsolver and Woods 1997a, Petersen et al. 2000).

Figure 18: *Manduca sexta* larval feeding activity under different light and temperature regimes. Damage to *Nicotiana attenuata* leaves by *Manduca sexta* larvae is roughly constant per unit initial larval mass under constant light and temperature conditions, but fluctuates under diurnal conditions. Larvae were 2nd to 3rd instar. Leaf area removed by five *M. sexta* larvae (leaf area in cm² g larval mass⁻¹ h⁻¹), under the three experimental light regimes, mean ± SE (n = 5-6). Damage rates per unit initial larval mass were relatively constant under constant light (LL) conditions or constant dark (DD) conditions, but fluctuated under diurnal (LD) conditions. Statistical analysis of the fluctuation under diurnal conditions was performed by Grit Kunert and is published here with her permission (see Herden et al. in prep. and methods section 2.11. for the final methods). Small letters a and b denote significant differences in damage rates between time-intervals. Damage per unit mass and time was significantly greater in the 2nd and 3rd time-intervals from 13:00 to 21:00 than in all other time-intervals under LD conditions. Time-intervals which did not significantly differ were grouped (small letters a and b) and compared in a linear mixed effects model (likelihood ratio test, P < 0.0001).
4. Discussion

From the results of this thesis may be concluded that the benefits of synchronous entrainment, demonstrated for plant resistance to *Trichoplusia ni* feeding on *A. thaliana* under laboratory conditions, do not appear in the *N. attenuata-M. sexta* plant herbivore pair. This might be due to a) *N. attenuata* plant physiology, b) *M. sexta* behavior and development, c) experimental conditions of constant or diurnal conditions, or d) a combination of these factors.

4.1. Performance of *M. sexta* larvae feeding on *N. attenuata* is not affected by out-of-phase entrainment

*M. sexta* larval performance was similar on in-phase and out-of-phase entrained *N. attenuata* plants (Fig. 8), under all of the three experimental set-ups, employing constant (LL or DD) or diurnal (LD) conditions (see experimental scheme in Fig. 4). However, larval development was slower under DD conditions than under LL and LD conditions, the most frequent instar at the end of the experiment being slightly younger and the slope of the mass increase less steep, under DD conditions (Fig. 8). Discrepancies in developmental rate between the other two light conditions and DD might have been due to slightly different temperature regimes, in the absence of the warming radiation of lamps (see e.g. different rate of mass increase under controlled temperature conditions of the feeding activity assay; table S2 in the supplemental data), less nutritious plant material due to carbon starvation (Fig. 16) and accompanying degradation processes (see discussion section 4.4.), or other unknown factors.

There is only one time-point where larvae on out-of-phase plants are significantly heavier than larvae on in-phase plants – on day 7 of the DD performance experiment (Fig. 8); but the difference is barely significant (p-value = 0.0293) and in magnitude below that of the experiment of Goodspeed et al. (2012). *Trichoplusia ni* larvae feeding on out-of-phase entrained *A. thaliana* plants for 3 days under DD conditions were already two to three times the average mass of larvae on in-phase control plants.
Discussion

(Goodspeed et al. 2012). It is the magnitude of the effect, in the *A. thaliana*-*T. ni* plant-herbivore pair, that lends it credibility and that makes it plausible that this is not just a transient difference, as the one observed in the DD experiment of larval performance (Fig. 8).

Some papers found differences between treatments in *M. sexta* only at the pupation state (e.g. Zavala et al. 2004). But larvae in LL and LD were already at the beginning of the 5th instar, the last instar, at the end of the performance experiment, and there were no obvious tendencies for different development between treatments, as there had been e.g. in Zavala et al. (2004), that would have spoken for an extension of the performance experiment.

There is, however, the possibility that performance of the specialist herbivore *M. sexta* was not affected by differences in plant resistance between in-phase and out-of-phase plants, especially if they are slight; *M. sexta* is, for instance, known to have a high tolerance for nicotine (Wink and Theile 2002). Therefore, to assess whether plant resistance was different in in-phase and out-of-phase plants, it was necessary to test a biochemical measure for the defense capability of *N. attenuata*: the induced responses to *M. sexta* herbivory in attacked rosette leaves.

4.2. In-phase and out-of-phase *N. attenuata* plants showed similar induced responses to *M. sexta* herbivory despite minor differences

*Nicotiana attenuata* has a rather effective constitutive defense, in the form of nicotine (Baldwin 1999, Steppuhn et al. 2004). However, it has come into use as a model plant for its pronounced induced responses to damage and herbivory, which are now well characterized in *N. attenuata* (e.g. Steppuhn et al. 2004, Heiling et al. 2010, Kaur et al. 2010, Gaquerel et al. 2010, Kim et al. 2011, Onkokesung et al. 2012, Woldemariam et al. 2013) In response to herbivory and detection of herbivore-specific elicitation cues (Bonaventure 2012) of the specialist herbivore *M. sexta*, a number of effective secondary metabolite accumulate (Glawe et al. 2003, Heiling et al. 2010, Kaur et al. 2010). Furthermore, in a tolerance response upon herbivory, plants might re-adjust their allocation of carbon resources, i.e. stronger carbon export to the roots (Schwachtje et al. 2006).
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In all three experimental conditions, metabolomics profiles of *N. attenuata* plants changed markedly after 3 d of feeding by *M. sexta* larvae, with little difference in the intensity of change between in-phase and out-of-phase plants (Fig. 9 and 10). As is evident from the principal component analyses in Fig. 9, plants of the in-phase and out-of-phase treatment started out clearly separated in LD and LL before herbivory, and had become more similar, after 3 days *M. sexta* herbivory under the respective light regime. Similarly, in DD, in-phase and out-of-phase plants had become even more similar after 3 days herbivory, although there was already a clear overlap of 95% confidence intervals before herbivory (Fig. 9). Moreover, as can be seen when metabolic profiles of time-points, before pre-treatment under constant conditions, are included in PCAs (Fig. 10), pre-treatment did not reduce differences between in-phase and out-of-phase plants, as originally intended. Rather, the pre-treatment enhanced differences and, after pre-treatment, the overlap between 95% confidence intervals of in-phase and out-of-phase plants was reduced, in LL and DD, compared to the conditions before pre-treatment, achieving the opposite of the intended (see results section 2.3.).

The overall similar induced response in in-phase and out-of-phase *N. attenuata* plants in response to 3 days herbivory of *M. sexta* (Fig. 9 and 10), is also mirrored by the results for the quantification of target secondary metabolites. All in all, plants of both treatments reacted similar to 3 days herbivory, and the strength of the induced defense response was much stronger affected by light conditions than by the prior entrainment regime (Fig. 11, 12, 13, 14 and 15).

While a few significant differences could be recovered between in-phase and out-of-phase plants, they should be viewed cautiously, as, in the same experiment, some significant differences occurred at unexpected times. For instance, in rutin and total CGA (Fig. 13 and 14), there were differences between treatments at the start of the experiment, in either DD or LL, respectively. While this is not surprising due to the different entrainment regime of in-phase and out-of-phase plants, this difference should have been recovered in LL and DD, respectively; both experiments were conducted simultaneously and received plants from the same in-phase and out-of-phase batches. Button et al. (2013) points out that statistical tests with small sample
size and, thus, low power, are less reliable, when it comes to non-significant results as well as, when a significant difference is recovered.

Other significant differences might have been more the result of treatment conditions than herbivory. For instance, the significant higher nicotine levels in in-phase plants under DD, started to appear after the DD pre-treatment and persisted (Fig. 11). Changes in levels of secondary metabolites that do not respond to *M. sexta* herbivory might indicate these effects of light conditions even better, i.e. falling total CGA and rutin levels in DD (Fig. 13 and 14) in the wake of carbon starvation (Fig. 16).

Some other differences are more difficult to explain, e.g. reductions in total CGA and rutin after herbivory in LL (Fig. 13 and 14). Remarkably, there were even results that were contrary to the hypothesis that out-of-phase plants are less well-defended than in-phase plants: Out-of-phase plants accumulated significantly higher levels of CP than in-phase plants, under LL conditions (Fig. 12).

Target secondary metabolites can only give a glimpse at possible differences in defensive capability and physiological differences between plants and should consequently not be over-interpreted. Still, it is remarkable that different defense metabolites react differently to various light conditions and that prior entrainment relative to herbivores appears not to play a major role. Here, the general metabolomics profiles of plant tissues (Fig. 9 and 10) might help to capture major trends despite small sample size, because a broad spectrum of metabolites might have more statistical power.

4.3. *Induced responses and light conditions*

Perception of light for entrainment to environmental conditions is perceived by photoreceptors, phytochromes and cryptochromes (Franklin and Quail 2010, McWatters and Devlin 2011)(see also introduction section 1.2.). Another function in biotic interaction is the detection of competition for light by neighboring plants (Franklin 2008, Keuskamp et al. 2010, 2012, Ballaré 2014). Proximity of potential competitors is sensed via the increase in reflected far-red radiation, which leads to a reduced red to far-red ratio (R:FR) in the incoming light, which then is perceived via
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Phytochromes, specifically phytochrome B. Actual shading is sensed via a number of different light signals, that come about by the reduction of radiation of certain wavelength, as the light penetrates through the canopy. Signals of actual shading include a reduced R:FR through the decrease of red light and increase in reflected far-red light. Furthermore, there is a reduction of blue light and, in natural settings, of UV light. The reduced R:FR is again perceived through phytochrome B, while the reduction of blue light is mainly perceived via cryptochromes (Ballaré 2014). If a plant, as a photosynthetic, sessile organism, is outgrown by competitors and shielded from photosynthetically active radiation, this can mean starvation and eventually the death of the plant (Ballaré 2014).

As previously mentioned, *N. attenuata* plants of the accession, used for my experiment, originated from an arid habitat, the sage-juniper habitats of the Great Basin desert (Baldwin 1999), where the plants grows standing solitary and unshaded (Fragoso, V., personal observation). Furthermore, the strong phenotypic response to DD conditions (see Fig. 17 and section 3.5.) indicates that *N. attenuata* has little tolerance for shading.

Interestingly, the prominent, reversible phenotypic change in *N. attenuata* rosette-stage plants in DD conditions (Fig. 17 and S5) includes vertical leaf movement (hyponasty). Hyponasty is part of a suite of phenotypic changes usually encountered in plants as a response to shading, the shade avoidance syndrome (SAS) (Franklin 2008, Keuskamp et al. 2010). In this case, it is probably better to talk about a SAS-like phenotype in *N. attenuata* rosette-stage plants under DD conditions, because in DD conditions the mode of shading perception is unclear. In constant darkness, the normal shading cues, an increased R:FR or depletion in blue-light wavelengths (Ballaré 2014), as they are employed in normal shading experiments (Pierik et al. 2004, Keuskamp et al. 2012), are absent. Furthermore, *N. attenuata* plants did not express other phenotypic changes that are part of the SAS, such as apical dominance, increased elongation or elongation of petioles, which are supposed to help plants outgrow competitors (Franklin 2008, Keuskamp et al. 2012), although this might have been due to the short time of DD exposure.

In this desert habitat, *N. attenuata* plants are exposed to high PAR irradiances (photosynthetically active radiation; wavelength between 400 and 700 nm; Ballaré
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For instance, Dinh et al. (2013) reported PAR fluences of 1300-1500 µmol m$^{-2}$ s$^{-1}$ as “typically measured on sunny days”, in the experimental field plot of the department for Molecular Ecology, near Lytle ranch, Utah (37° 8' 36" north latitude, 114° 1' 20" west longitude; http://wchsutah.org/homes/lytle-ranch.php). On the other hand, in the climate chambers, PAR irradiance was about ~400 µmol m$^{-2}$ s$^{-1}$, at the top of the potted plants, and thus considerable lower than under natural conditions. This might be the reason why LL conditions did not result in visible stress or discernible phenotypic changes (e.g. chlorosis or necrotic tissue, Sysoeva et al. 2010)(see Fig. 17 and S5) in the *N. attenuata* rosette-stage plants.

In constant light conditions, a higher accumulation of HGL-DTGs after 3 days herbivory was observed than under diurnal conditions (Fig. 15). Similarly, after herbivory in LL, out-of-phase plants accumulated CP above levels encountered in in-phase plants of the same experimental set-up, or in the diurnal experimental set-up, in both treatments (Fig. 12). This comparison is tentative, because plants from the diurnal experiment were slightly older at the initiation of herbivory, plant batches were not identical and experiments were conducted at different seasons (see methods section 2.3.). Non-the-less, there is no literature that indicates a connection between increased PAR light availability and increased levels in these secondary metabolites. While UV-B radiation (wavelengths between 280 and 315 nm; Dinh et al. 2013) is a well-known stress factor for plants (e.g. Chimphango et al. 2007), has been shown to increase accumulation of phenolics in plants (Izaguirre et al. 2007, Demkura et al. 2010), and increases HGL-DTG accumulation in *N. attenuata* (Dinh et al. 2013), the lamps in the climate chamber emit hardly any light in the UV-B range, ruling out this factor for my LL experiment (see supplementary figure S1 for a representative light spectrum).

The reversible SAS-like phenotype change in *N. attenuata* plants (Fig. 17 and S5) might indeed indicate that plants perceive the constant darkness conditions in a similar manner, as they perceive shading. SAS phenotypes can be induced by both a reduction of blue light wavelengths from the incoming light spectrum or a low R:FR in the incoming light (Ballaré 2014). A blue light depletion could simply be “sensed” by the complete absence of blue light, while a low red to far-red ratio is far more difficult to “simulate”. A low red to far-red ratio is perceived mainly by the
photoreceptor phytochrome B. It is biosynthesized in its inactive \( P_r \) form and is activated to its active \( P_{fr} \) form, by red wavelength (660 to 670 nm), but can be deactivated again by far-red light (725 to 735 nm). Far-red light re-converts the \( P_{fr} \) form to the inactive \( P_r \) form (Franklin 2008). Thus, a low R:FR in incoming light directly translates to a high \( P_r : P_{fr} \) ratio (Franklin 2008). In DD conditions, a similar high \( P_r : P_{fr} \) ratio could be brought about by passive accumulation of biosynthesized \( P_r \) (Franklin 2008), when activation fails under DD conditions, and both \( P_r \) and \( P_{fr} \) continue to be degraded in the normal manner.

A high \( P_r : P_{fr} \) ratio in phytochrome B under DD conditions could furthermore be of significance for the expression of defense under DD conditions, since a number of studies have shown that jasmonate-dependent defense strategies can be down-regulated when plants are exposed to a low R:FR (Cerrudo et al. 2012, Izaguirre et al. 2013). Furthermore, shading can reduce the accumulation of bioactive jasmonates (Agrawal et al. 2012). It has been hypothesized (Ballaré 2014) that a low R:FR leads to the down-regulation of jasmonate signaling in plants, via the inactivation of phytochrome B, expressed in the high \( P_r : P_{fr} \) ratio. This might be a possible mechanism that explains, at least partially, why shaded plants and plant parts show less plant resistance to pathogens and herbivores (as seen in several studies; see Ballaré (2014) for review) and, in my case, a possible mechanism that might explain why induced responses were reduced under DD conditions.

Specifically, in constant dark conditions, I observed a reduced separation of metabolomics profiles before and after 3 days \( M. sexta \) herbivory (Fig. 9), when compared to LL and LD conditions, indicating that the induced responses to herbivory were less pronounced under DD conditions. Furthermore, HGL-DTGs failed to accumulate in response to herbivory under DD and rather remained near constitutive levels, even slightly dropping with time in constant darkness (Fig. 15). Moreover, CP failed to accumulate to quantifiable levels after herbivory in DD (Fig. 12). However, what contradicts such a presumed down-regulation of jasmonate signaling in constant darkness is the fact that nicotine accumulation, which has repeatedly been shown to be jasmonate-regulated in \( N. attenuata \) (Baldwin 1999, Halitschke et al. 2000), was not affected by DD conditions.
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4.4. *Induced defense responses and carbon balance*

It would be easy to draw a connection between the amount of carbon assimilated and the amount of carbon that can be invested in defense metabolite production, as when secondary metabolites with a high portion of carbon atoms, here HGL-DTGs, appear to show a strong response to light availability (Fig. 15). As stated above, light of certain wave lengths can directly modulate the defense response or the accumulation of defense-related secondary metabolites (Ballaré 2014). This is why the question for carbon reserves as reason for different accumulation of secondary metabolites should only be asked secondarily. The carbon-nutrient balance hypothesis has been repeatedly criticized for trying to draw a connection between the amount of available nitrogen and carbon and which nitrogen-rich or carbon-rich compounds should be deployed by the plant (Stamp 2003).

For my experimental setting, modulation of defense by light of certain wavelengths cannot explain the accumulation of HGL-DTGs and CP after 3 d herbivory, under LL conditions, to levels higher than under LD conditions (Fig. 12 and 15). It might be reasonable to ask for secondary factors, such as available energy in terms of carbon reserves or even amount of available photosynthetic assimilate as biosynthetic precursors. Both HGL-DTGs and CP are connected to biosynthetic pathways in chloroplasts of leaf tissue (Taiz and Zeiger 2006). The diterpene backbone of HGL-DTGs is, at least partially, synthesized by the plastidial terpene biosynthesis pathway (Heiling et al. 2010), and CP is synthesized from precursors from the plastidial shikimate pathway (Taiz and Zeiger 2006). Thus, production of both compounds might be connected to the availability in photosynthetic assimilate in chloroplasts or the energy status in chloroplasts. Concerning the status of carbon reserves, stored and transported as sugars and starch, there might be a more direct link to HGL-DTG biosynthesis. The diterpene backbone is conjugated to sugar moieties (Heiling et al. 2010) which should be available in excess under LL conditions, as starch accumulated to higher levels in LL (Fig. 16) than normally found in *N. attenuata* plants at the end of a 12h day, and the levels of soluble sugars were similarly elevated (Fig. S2, S3 and S4). But whatever higher levels in certain secondary metabolites plants in LL might have accumulated over plants in LD
conditions, *M. sexta* larvae developed at a similar rate on plants of the LD and the LL performance assay (Fig. 8).

Depletion of starch levels as well as soluble sugar levels in rosette leaves, during the time in DD conditions (Fig. 16, S2, S3 and S4), is a strong indicator for carbon starvation in *N. attenuata* plants. Carbon starvation, when prolonged, has several severe consequences for plants. In *A. thaliana* plants, carbon starvation leads to a reversible growth arrest and, when maintained for several hours, to transcriptional activation of genes involved in the reaction to carbon starvation. With time in DD, alternative energy sources are used and degraded, such as proteins, cell walls and lipids in autophagic processes (Gibon et al. 2004, Bläsing et al. 2005, Smith and Stitt 2007, Usadel et al. 2008). Next to the break-down of alternative energy sources, such as proteins, there may also be degradation of plastids (Wittenbach et al. 1982, Brouquisse et al. 1998). When darkness is extended for very long time (e.g. more than 8 days in rather resilient peal millet seedlings), necrosis of plant tissues follows (Baysdorfer et al. 1988).

In line with this degradation processes is the observation that rosette leaves in *N. attenuata* seemed to become thinner and the leaf material was more likely to tear, with time in DD conditions (personal observation during collection of leaf material). Moreover, the weakened induced response to *M. sexta* herbivory in DD conditions (Fig. 9 and 10) might very well be due to these consequences of carbon starvation. Among the quantified secondary metabolites, total CGA and rutin, both compounds that usually do not respond to *M. sexta* herbivory (Keinänen et al. 2001), decreased in *N. attenuata* plants with time in DD (Fig. 13 and 14), perhaps due to these degradation processes. Similarly, levels of HGL-DTGs fell with time in constant darkness, to levels even below constitutive levels after 4 days in DD (Fig. 15), although HGL-DTGs had been expected to increase in the course of *M. sexta* herbivory (Heiling et al. 2010). Furthermore, after 3 days *M. sexta* herbivory under DD, CP had not accumulated to quantifiable levels (Fig. 12). The failed accumulation of HGL-DTGs and CP after herbivory might be directly connected to degradation processes, if these processes should comprise degradation of proteins of biosynthetic machinery or degradation of plastids and thus biosynthesis of the compounds. Another direct connection between failed accumulation in HGL-DTGs
and carbon starvation might be a lack of sugar moieties for biosynthesis (Heiling et al. 2010). Alternatively, the biosynthesis of secondary metabolites might be inhibited in leaf tissue in the course of the starvation response, similar to the growth arrest in carbon-starved *A. thaliana* plants (Smith and Stitt 2007).

Surprisingly, nicotine accumulation (Fig. 11) was less affected by carbon starvation or the presumed accompanying degradation processes, maybe hinting at a tissue-specific response to carbon starvation, as nicotine is synthesized exclusively in the roots of *N. attenuata* (Baldwin 1999). The accumulation of nicotine after mechanical damage, via sampling, and *M. sexta* herbivory proved relatively robust under the three different light regimes (LL, DD and LD)(Fig. 11), despite different status in carbon reserves in the plants. However, the 24h DD pre-treatment appears to have generated differences in nicotine accumulation between in-phase and out-of-phase plants (Fig. 11), that themselves could be due to the status of carbon reserves at time of transfer to DD (end of day vs. end of night; see Fig. 4). Interestingly, nicotine accumulation has also proved to be somewhat resistant against nitrogen deprivation (Lynds and Baldwin 1998).

The weakened induced response of *N. attenuata* rosette-stage plants of both treatments, under DD conditions (Fig. 9, Fig. 12 and Fig. 15), compared to diurnal conditions, raises the question, how the strongest indicator for reduced plant resistance in out-of-phase *A. thaliana* plants could be found under DD conditions (Goodspeed et al. 2012). In DD conditions, plants might be compromised due to carbon starvation (Graf et al. 2010) and not make for the most nutritious food plants (Fig. 8).

Out-of-phase *N. attenuata* plants, under diurnal conditions, could have been expected to be compromised in their carbon balance, as they adjusted to the in-phase light regime; but, since out-of-phase plants had experienced a 12h day before the transfer to the in-phase light regime at 9 am, and this was followed by another 12h day, out-of-phase plants probably accumulated excess starch, as did plants under LL conditions. Maybe these improved carbon reserves aided out-of-phase plants when faced with the physiological stresses of re-entrainment.
Theoretically, excess and depletion of carbon resources in both treatments, in LL and DD conditions, respectively, might have similarly masked the effect of out-of-phase entrainment under constant conditions. However, most evidence points to that that out-of-phase entrainment does not reduce the induced response to *M. sexta* and, thus, the degree of plant resistance, compared to in-phase plant. The induced response to herbivory proved rather plastic to environmental conditions and potential energy constraints.

### 4.5 Fluctuations in *M. sexta* feeding activity are probably brought about by alternating day-night regimes

Finally, after the effects of plant physiology in *N. attenuata* and experimental conditions that might negate this effect of enhanced plant resistance through in-phase entrainment, the variable “herbivore” needed to be considered.

In their natural environment, *M. sexta* larvae employ the strategy of sitting hidden under the leaves of the host plants, utilizing their green camouflage and slow movements to remain inconspicuous (Casey 1976). Larvae, furthermore, utilize their host plant for shade in the strong temperature fluctuations of the desert, as their upper lethal temperature turned out to be 45 °C (Casey 1976). However, feeding activity and growth of the *M. sexta* larvae show a strong temperature dependence; growth and consumption rates increase between 14 and 26 °C and had their maximum close 34 °C, when larvae were kept under laboratory conditions (Kingsolver and Woods 1997).

It was therefore less surprising that *M. sexta* larvae under constant conditions showed an arrhythmic behavior, while larvae reacted strongly to the temperature fluctuations under diurnal conditions and displayed a significant difference in damage rate between time-intervals of day and night (Fig. 18). Depending on temperature fluctuations in its habitat the lepidopteran could then be predictably rhythmic to times of year with higher temperature fluctuations throughout the day, and arrhythmic when temperatures allow feeding at a similar rate throughout the day.

Ultimately, activity patterns of animals are plastic and are subject to modifications (Yerushalmi and Green 2009); by e.g. environmental conditions (van Oort et al. ...)
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2005, 2007), social cues (Riccio and Goldman 2000, Bloch et al. 2001, Shemesh et al. 2010) or even absence or presence of predators and competitors (Aviv 2005, Fenn and Macdonald 2014). While herbivores with a predictable circadian activity pattern, as in T. ni, might actually exist, plants like N. attenuata are confronted with many different herbivores (Schuman et al. 2013) that likely have very different activity patterns; and adaptation to a particular pattern, especially in a generalist herbivore, sounds unlikely.

4.6. Optimized defense patterns on different time-scales

Optimal defense strategies in plants should take into account predation risk, costs of defense, benefits of defense and the value of the plant tissue, the defense is applied to (Stamp 2003, Meldau et al. 2012). Phytohormones allow the fine-tuning of these defense strategies, particularly jasmonates, when it comes to anti-herbivore strategies (Wasternack 2007, Meldau et al. 2012, Meldau and Baldwin 2013, Wasternack and Hause 2013). If stressful events repeat predictably within a 24h day, it can make sense to utilize the circadian clock for the anticipation of these events and prepare accordingly (Sanchez et al. 2011, Wang et al. 2011, Zhang et al. 2013). The predictable feeding activity maximum of T. ni (Goodspeed et al. 2012) might constitute such an example, and, if other herbivores, A. thaliana is confronted with in its habitat, display similar activity maxima during the day, it might be useful to be constitutively more alert during the day with higher jasmomate levels (Goodspeed et al. 2012). However, jasmonate accumulation can constrain growth processes (Wasternack and Hause 2013) and interaction with other phytohormones might actually be the reason for the peaking time of the jasmonate accumulation pattern in A. thaliana. Moreover, it is plausible that defenses can be optimized on different time-scales. If a defense can be directly deployed as e.g. extrafloral nectar in lima beans (Radhika et al. 2010), a daytime specific utilization is possible. However, induced defenses take several days to accumulate in N. attenuata (e.g. Halitschke et al. 2000, Gális et al. 2010), making a daytime-specific utilization unlikely, and the relaxation is on a similar time-scale of days in CP, when simulated herbivory is applied (Gális et al. 2010). Again, other defense patterns are more extreme and span several generations like the transgenerational priming of defense responses in A. thaliana (Rasmann et al. 2012).
4. 7. Conclusions

When tested under three different light regimes (LL, DD and LD), out-of-phase entrainment of *N. attenuata* rosette-stage plants had no sustained positive effect on performance of *M. sexta* larvae. Nor was there a pronounced effect on plant resistance, in-phase and out-of-phase entrained plants showing a similar degree of induced responses to 3 days of *M. sexta* herbivory. Moreover, it became apparent that constant conditions (LL and DD) had profound effects on the expression of defense in *N. attenuata* plants; specifically, there were several strong indicators for stress under constant darkness.

On the one hand, my results show that circadian clock experiments with plants should be avoided under DD. Maybe plants should rather be exposed to constant low light levels to avoid complete carbon starvation (Haydon et al. 2013). However, some plants might be more sensitive to LL than others, and the same probably holds true for DD, depending on shade tolerance and light tolerance of the respective plant species.

It specifically raises the question in-how-far the results of Goodspeed et al. (2012) were affected by such light effects, as their most pronounced effects were found under DD conditions. On the other hand, the question arises whether effects of light regimes have masked differences in defense capability between in-phase and out-of-phase *N. attenuata* plants, by providing energy in excess under the one condition and starving plants completely under the other condition. However, the results from the experimental set-up with “jet-lagged” plants indicate that out-of-phase entrained plants still had a similar capability for induced responses, under diurnal conditions, without the effects of constant conditions. This is my strongest support for the conclusion that this effect of enhanced plant resistance through synchronous entrainment probably does not exist in the *N. attenuata-M. sexta* plant-herbivore pair.

In the end, the neglectable effect of out-of-phase entrainment on plant resistance might also have been brought about by the arrhythmic behavior of *M. sexta* larvae, under constant conditions, or too small fluctuations in feeding activity under diurnal conditions.
However, *M. sexta* larvae develop rather rapidly and damage rates increased with caterpillar growth, visibly increasing within 24h. Accounting for this increase in damage rates, diurnal fluctuations in *M. sexta* activity might have less ecological relevance, as effective defense needs to be set-up before larvae show their full potential for damage. Furthermore, induced defense metabolites accumulate over several days, and increase even more if damage is sustained, meaning that effective defense is already set-up belated, making an “as-fast-as-possible” accumulation in secondary metabolites more plausible than one with different increments at different times of day, although these might then rather be related to physiological constraints.
Summary

5. Summary

The circadian clock allows the synchronization of plant physiology with predictable events, brought about by the rotation of Earth on its axis. The clock specifically permits the anticipation of such events which include e.g. dawn, abiotic and, as recently demonstrated, biotic stress factors. A recent study showed that plants may be able to anticipate, and prepare for daytime-specific maxima of herbivore attack. In this specific case, Goodspeed et al. (2012) were able to show that Arabidopsis thaliana plants had reduced resistance against Trichoplusia ni larvae, when plants and larvae were co-incubated under constant conditions and plants had previously been entrained under a 12h light:12h dark cycle, with a phase 12h off from the entrainment regime of the larvae.

T. ni showed circadian fluctuation in feeding activity, and, because constitutive and induced, circadian fluctuating jasmonate phytohormone levels in A. thaliana peaked before the time of maximal activity, it was hypothesized that thus might confer a faster reaction to cues of herbivore attack at this subjective daytime via increased expression of jasmonate-regulated genes. Jasmonate signaling controls a major pathway in induced defense responses in plants.

The aim of this thesis was to test for a similar effect in another plant-herbivore pair; specifically, if asynchronous entrainment of host plants and herbivores under opposite diurnal regimes leads to a weaker defense response in the wild tobacco Nicotiana attenuata to its co-evolved specialist herbivore Manduca sexta than prior synchronous entrainment under identical diurnal regimes. This was tested by co-incubation under constant conditions (LL and DD) and diurnal conditions, after prior identical or opposite entrainment; larvae infested host plants for 2-3 days.

Measured plant resistance parameters were the growth of M. sexta larvae and the induced defense response of attacked N. attenuata plants, detected via untargeted metabolite profiles and quantification of target secondary metabolites. Surprisingly, the respective entrainment treatment did not sustainably affect either M. sexta growth or strength of the induced defense response, under any of three light conditions. What is more, that constant conditions were stressful for plants became apparent
Summary

under constant dark conditions, which reduced the magnitude of plants’ inducible response, accompanied with shade avoidance syndrome-like symptoms and carbon starvation. However, since damage rates of *M. sexta* larval feeding increased in a similar manner as larval growth, it might also be hypothesized that there is no adaptive value in anticipation of small-scale diurnal fluctuations against a background of rapidly increasing damage, where fast induced defense or tolerance responses can make all the difference.
6. Zusammenfassung


Zur Überprüfung des Verteidigungszustandes der Pflanzen wurden einerseits die Gewichtszunahme der Raupen als auch die direkte Verteidigungsausprägung in der
Zusammenfassung

Pflanze untersucht; letzteres geschah, indem man das unspezifische Metabolit-Profil von Blattmaterial vor und nach Raupenfraß untersuchte. Des Weiteren wurde die Konzentration einer kleinen Anzahl von Sekundärmetaboliten im Blattmaterial quantifiziert, für die eine physiologische Wirkung auf die Entwicklung von *M. sexta*-Raupen nachgewiesen worden ist.

Schlussendlich wurde unabhängig vom Entrainment der Pflanzen eine vergleichbare Ausprägung der induzierten Verteidigungsantwort gefunden. Vielmehr hatten Umweltbedingungen eine Auswirkung auf die Stärke der induzierten Antwort; wohlmöglich besonders unter konstanter Dunkelheit aufgrund des entstehenden Mangels an Kohlenstoffreserven. Es kann nicht klar entschieden werden, ob a) ein messbarer Effekt des Entrainment auf die Pflanzenresistenz ausbleibt, weil das Fehlen rhythmischer Herbivorenaktivität unter den gegebenen Experimentbedingungen von LL und DD, es der Pflanze unmöglich macht, die Fraßaktivität der Raupen vorherzusagen, oder ob b) eine derartige Vorhersage in der freien Natur vielleicht nur einen geringen ökologischen Fitnessvorteil bringt, angesichts des schnellen Wachstums von *M. sexta*-Raupen und der im Verhältnis dazu geringen tageszeitlichen Schwankungen in der Aktivität. Im zweiten Fall würde es dann vielmehr Sinn machen zu versuchen, so schnell wie physiologisch möglich eine entsprechende Verteidigungs- oder Toleranzantwort zu induzieren.
Acknowledgements

7. Acknowledgements

I would like to thank my supervisors, Prof. Ian Thomas Baldwin, for supporting this project and for giving me the opportunity to turn this thesis into a publication, and Prof. Stefan Halle, for accepting this external project.

This project would not have been possible without support from my direct supervisors Dr. Meredith Schuman, Dr. Stefan Meldau and Dr. Sang-Gyu Kim, who helped guide this project and were foremost in helping design experiments and interpreting results. Regarding the practical part of the project, I am deeply indebted to the technical assistants and technicians of our department for their patience and their instructions on several biochemical methods as well as maintaining the analytical equipment in a “tip-top” state. I would also like to thank the gardeners for taking such good care of my plants and even putting up with the rather unorthodox conditions during our constant dark experiment; furthermore, thanks to Daniel Veit for providing us with lamps and curtains on such short notice. I thank all Clockwork Green members for lending a hand during my experiments.

I had a great time in this department, thanks to a lot of great people, especially Arne, Mario, Cristina, Felipe, Heidi, Lucas and Martin, some of whom kept me well-fed with chocolate. Merry, I would like to thank you personally as a friend for patiently waiting for me finally finishing all the Hausarbeiten in my final year of studies and giving me a lot of input for good scientific practice and improving my writing skills.

I would like to thank my parents for their emotional and, not to mention financial support, in my rather lengthy biology studies. I especially would like to dedicate this work to my grandparents who never ceased to ask for me finishing and my late grandmother who died last year and about whom I knew much too little. Secondly, I am really grateful to all those friends I found in these seven awesome years of my studies in Jena and their emotional support in difficult phases of my studies: I am going to miss you, especially Eva and Elisa, my former roommates, as we now go about finding our first jobs and leaving Jena, probably for good.
8. References


Kaur, H. et al. 2010. R2R3-NaMYB8 regulates the accumulation of phenylpropanoid-polyamine conjugates, which are essential for local and systemic defense against insect herbivores in *Nicotiana attenuata*. - Plant Physiol. 152: 1731–47.


References


References


9. Declaration of authorship

I confirm herewith that I have written this thesis by myself and that I did not use any other sources and assistance except the ones indicated and that I have referenced citations and mentally transferred contents.

Jena, September 2014  

……………………

(signature)

Selbstständigkeitserklärung

Hiermit versichere ich, dass ich die vorliegende Arbeit selbstständig verfasst und keine anderen als die angegebenen Quellen und Hilfsmittel verwendet habe.

Jena, September 2014  

…………………………

(Unterschrift)
10. Supplemental figures and tables

**Supplemental figure 1:** Representative spectrum of light sources in the climate chambers. The spectrum of the lamps in the York chambers was recorded by the technicians of the MPI for Chemical Ecology. The figure is shown here with permission. As already stated in the methods, climate chamber lamps were the same as in the glasshouse, Master Sun-T PIA Agro 400 W sodium lights (Philips, Amsterdam, Netherlands), 100% intensity setting, supplemental light with 1000-1300 µmol m⁻² s⁻¹ photosynthetic photon flux density supplied by 450 W Na-vapor high-intensity discharge bulbs (from Master Sun-T PIA Agro 400-W sodium lights, Philips, Amsterdam, Netherlands). This spectrum shows hardly any emittance in the UV-A range (315 to 400 nm) or shorter wavelengths, but pronounced peaks infrared range (700 to 1000 nm), and of course considerable peaks in the PAR range (400 to 700 nm).
Materials and methods

(See next page for figure caption.)
Supplemental figure 2: Accumulation of glucose in leaves of \textit{N. attenuata} rosette stage plants after 3 days \textit{M. sexta} herbivory under different light regimes. Plant material was collected at three time-points under DD and LL (0 d in continuous conditions, 1 d in continuous conditions, and 4 d in continuous conditions [= 3 d herbivory under continuous conditions]) and two time-points under diurnal conditions (LD) (0 d of herbivory, and 3 d of herbivory). Relative contents of glucose were compared between in-phase and out-of-phase plants of a time-point by either student t-tests or Wilcoxon rank sum tests for independent samples (see text in results section 3.4 for the respective details). Number of samples, n = 5 for LL, n = 3-5 for DD, and n = 3-4 for LD conditions; but see text in results section 3.4 for details. Note that because the soluble sugar data is rather preliminary, a number of sample groups were not quantifiable ( n < 3) or a number of time-points could not be tested, because n < 4 in at least one of the two treatments. Stars denote significant differences between in-phase and out-of-phase plants (* - p-value < 0.05, ** - p-value < 0.01, *** - p-value < 0.001).
Materials and methods

(See next page for figure caption.)
Supplemental figure 3: Accumulation of fructose in leaves of *N. attenuata* rosette stage plants after 3 days *M. sexta* herbivory under different light regimes. Plant material was collected at three time-points under DD and LL (0 d in continuous conditions, 1 d in continuous conditions, and 4 d in continuous conditions [= 3 d herbivory under continuous conditions]) and two time-points under diurnal conditions (LD) (0 d of herbivory, and 3 d of herbivory). Relative contents of fructose were compared between in-phase and out-of-phase plants of a time-point by either student t-tests or Wilcoxon rank sum tests for independent samples (see text for the respective details). Number of samples, *n* = 4-5 for LL, *n* = 3-5 for DD, and *n* = 4-5 for LD conditions; but see text in results section 3.4 for details. Note that because the soluble sugar data is rather preliminary, a number of sample groups were not quantifiable (*n* < 3) or a number of time-points could not be tested because *n* < 4 in at least one of the two treatments. Stars denote significant differences between in-phase and out-of-phase plants (*p*-value < 0.05, **p*-value < 0.01, ***p*-value < 0.001).
Materials and methods

(See next page for figure caption.)
Supplemental figure 4: Accumulation of sucrose in leaves of *N. attenuata* rosette stage plants after 3 days *M. sexta* herbivory under different light regimes. Plant material was collected at three time-points under DD and LL (0 d in continuous conditions, 1 d in continuous conditions, and 4 d in continuous conditions [= 3 d herbivory under continuous conditions]) and two time-points under diurnal conditions (LD) (0 d of herbivory, and 3 d of herbivory). Relative contents of sucrose were compared between in-phase and out-of-phase plants of a time-point by either student t-tests or Wilcoxon rank sum tests for independent samples (see text for the respective details). Number of samples, n = 5 for LL, n = 3-5 for DD, and n = 7 for LD conditions; but see text in results section 3.4 for details. Note that because the soluble sugar data is rather preliminary, a number of sample groups were not quantifiable (n < 3) or a number of time-points could not be tested because n < 4 in at least one of the two treatments. Stars denote significant differences between in-phase and out-of-phase plants (* - p-value < 0.05, ** - p-value < 0.01, *** - p-value < 0.001).
Supplemental figure 5: Phenotypes of plants after exposure to constant conditions are reversible. After return to diurnal conditions, photos were taken of plants, again, 5 days later. Exposure to LL conditions did not delay elongation, but might have rather accelerated it. Exposure to DD conditions appears to have slightly delayed elongation. Photos were taken by Jasmin Herden.
Supplemental table 1: Average day and night temperatures in the York climate chambers. Mean temperatures (in °C) measured using temperature/light data loggers at four different positions, distributed in climate chambers used for Manduca sexta performance experiments (methods section 3.3), over at least 2 d, logging every 10 min. Mean day and night temperatures from the climate chamber with the in-phase light regime were used for diurnal (LD) feeding damage assays, while mean day and night temperatures from both chambers were used for constant light (LL) and constant dark (DD) feeding assays, respectively, since constant conditions had been achieved by transferring plants and larvae between the two climate chambers.

(See next page for table S1.)
## Materials and methods

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**Supplementary table S2: Caterpillar mass of replicates for quantification of leaf area removed.**

Masses (in mg) of *Manduca sexta* larvae used for feeding damage experiments (Figure 18). Each replicate (n) is the mean of 5 larvae. Exp, experiment; LL, constant light; DD, constant dark, LD, diurnal. The time-points give the times at which the larvae were weighed, at the start and at the end of the respective time-interval.

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