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Shifting *Nicotiana attenuata*'s diurnal rhythm does not alter its resistance to the specialist herbivore *Manduca sexta*

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Abstract

Arabidopsis thaliana plants are less resistant to attack by the generalist lepidopteran *Trichoplusia ni* when plants and herbivores are entrained to opposite, versus identical diurnal cycles and tested under constant conditions. This effect is associated with circadian fluctuations in levels of jasmonic acid, the transcription factor MYC2, and glucosinolate contents in leaves. We tested whether a similar effect could be observed in a different plant-herbivore system: the native tobacco *Nicotiana attenuata* and its co-evolved specialist herbivore, *Manduca sexta*. We measured larval growth on plants under both constant and diurnal conditions following identical or opposite entrainment, profiled the metabolome of attacked leaf tissue, quantified specific metabolites known to reduce *M. sexta* growth, and monitored *M. sexta* feeding activity under all experimental conditions. Entrainment did not consistently affect *M. sexta* growth or plant defense induction. However, both were reduced under constant dark conditions, as was *M. sexta* feeding activity. Our data indicate that the response induced by *M. sexta* in *N. attenuata* is robust to diurnal cues and independent of plant or herbivore entrainment. We propose that while the patterns of constitutive or general damage-induced defense may undergo circadian fluctuation, the orchestration of specific induced responses is more complex.

Keywords

circadian clock; diurnal rhythm; *Manduca sexta*; *Nicotiana attenuata*; plant-herbivore interactions

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Conflict of Interest

Ian T. Baldwin is an Associate Editor of the *Journal of Integrative Plant Biology*.

Theme: Plant-environmental interactions

Introduction

Through the Earth's rotation, day and night alternate in a ca. 24 h diurnal cycle. Most organisms have developed an internal circadian clock in order to synchronize their activities with predictable diurnal events (reviewed in Bell-Pedersen *et al.* 2005). Plants have been shown to gain a fitness advantage from having a circadian clock synchronized with their external environment (reviewed in Yerushalmi and Green 2009). Furthermore, some plant responses to abiotic stress (reviewed in Sanchez *et al.* 2011) and to biotrophic pathogen attack (Bhardwaj *et al.* 2011; Wang *et al.* 2011; Zhang *et al.* 2013) are mediated by the circadian clock, but less is known about plant responses to herbivore attack.

Goodspeed *et al.* (2012) showed that larvae of the generalist lepidopteran *Trichoplusia ni* have a circadian feeding pattern when observed on artificial diet. They found that *T. ni* entrained to a 12 h:12 h light:dark (LD) cycle grew larger and caused more damage on *A. thaliana* plants which had been entrained to a diurnal regime opposite that of larvae (out-of-phase), versus when plants and larvae were entrained to the same regime (in-phase). These experiments were performed under constant dark (DD) or constant light (LL) conditions, setups classically used in order to separate circadian from diurnal phenomena (Harmer 2009). Similar effects could be demonstrated for harvested fruits and vegetables from several plant families entrained under 12 h:12 h LD cycles prior to, or during infestation by *T. ni* (Goodspeed *et al.* 2013b). As had been shown for pathogen attack (Bhardwaj *et al.* 2011; Wang *et al.* 2011; Zhang *et al.* 2013), Goodspeed *et al.* (2012, 2013a) furthermore showed that in-phase plants of arrhythmic *A. thaliana* mutant lines (*CCA1-OX* and the *LUX ARRHYTHMO* knockout *lux2*) were not better defended than out-of-phase plants of the same lines under DD or LL conditions, implicating the clock in the anticipation of defense to *T. ni*. Additionally, jasmonic acid (JA) and salicylic acid (SA) levels fluctuated constitutively in *A. thaliana* plants under DD and LL, in oppositely phased circadian cycles, and *A. thaliana* lines deficient in jasmonate biosynthesis (*allene oxide synthase* [*aos*], *jasmonate resistant 1* [*jar1*]) were not better defended when entrained in-phase with *T. ni* (Goodspeed *et al.* 2012). This consistent effect of various plant clocks on the rhythmically feeding *T. ni* is intriguing, albeit difficult to understand in an evolutionary context, given that *T. ni* has rarely been observed feeding on *A. thaliana* in nature (Jander 2012).

In a follow-up study, Goodspeed and colleagues (2013b) demonstrated circadian fluctuation of glucosinolate contents in *A. thaliana* leaves as well as in harvested cabbage, with glucosinolate contents peaking during the day at levels threefold the nighttime trough. These fluctuations had a similar phase to the constitutive JA fluctuations demonstrated in the 2012 study and were proposed as a direct, if not complete, explanation of synchronized plants' superior resistance to *T. ni*, since this effect is likely to be mediated by JA-induced metabolites and not directly by JA (but see Kallenbach *et al.* 2012). However, the *A. thaliana myb28myb29* mutant, which does not produce aliphatic glucosinolates, also displayed greater resistance to *T. ni* after co-entrainment of plants and larvae, although overall resistance of *myb28myb29* was reduced in comparison to WT.

Thus the most straightforward interpretation of the studies by Goodspeed and colleagues is that circadian fluctuation in JA-mediated defenses allows plants to anticipate herbivore

attack. However there are other interpretations, and the “anticipatory defense” hypothesis suffers from some weaknesses: unlike foliar pathogens, herbivores do not depend on a circadian cycle of stomatal opening for attack. Even herbivores from the same family, sharing the same host plant, and sensitive to overlapping suites of jasmonate-mediated defenses may attack at different times of day, although individual herbivores do have characteristic activity patterns; and herbivore population dynamics are unpredictable (Denno *et al.* 1995; Jander 2012; Wigglesworth 1972).

We thus asked whether anticipatory defense can be observed in other plant-herbivore pairs (Jander 2012). Specifically, we tested whether the resistance of the wild tobacco *Nicotiana attenuata* Torr. ex. Watson (Solanaceae) to its specialist herbivore *Manduca sexta* Linne (Sphingidae) is also reduced when plants and herbivores are entrained out-of-phase with each other. *M. sexta* is both among the most damaging herbivores of *N. attenuata*, as well as an important pollinator (Kessler 2012). The intertwined evolutionary history of these two organisms should have afforded the opportunity for *N. attenuata* to anticipate attack by *M. sexta* larvae using any robust and expedient means.

We tested the hypothesis that out-of-phase entrainment of *N. attenuata* increases *M. sexta* growth and decreases *N. attenuata*’s elicitation of defense compounds under constant or diurnal experimental conditions. In designing our experiments, we considered that constant conditions can have detrimental effects on plant physiology: physiological disorders and photodamage in constant light (reviewed in Sysoeva *et al.* 2010), or carbon starvation in constant dark (e.g. Graf *et al.* 2010). In order to demonstrate a direct mechanism for the observed growth of *M. sexta* on differently entrained plants, we analyzed leaf material for general metabolomic fingerprints as well as target secondary metabolites before and after *M. sexta* feeding for all experimental set-ups, rather than analyzing levels of defense signaling hormones. *N. attenuata* accumulates a panoply of defense compounds in shoot tissue in response to *M. sexta* feeding, which have well-characterized effects on *M. sexta* growth rates. These include hydroxygeranyllinalool-diterpene glycosides (HGL-DTGs, Heiling *et al.* 2010), phenolics and putrescine conjugates (Kaur *et al.* 2010), and trypsin protease inhibitors (Zavala and Baldwin 2004). Although *M. sexta* is tolerant to high levels of nicotine, its growth is also reduced on nicotine-containing compared to nicotine-deficient plants, likely due to the metabolic cost of detoxification for larvae (Steppuhn *et al.* 2004).

We observed no difference in either *M. sexta* growth or *N. attenuata*’s elicitation of defense compounds as a result of synchronous versus asynchronous entrainment of plants and larvae under diurnal (LD) conditions; a small increase in production of defense compounds by asynchronously entrained plants and no difference in *M. sexta* growth under constant light (LL); and a transient increase in *M. sexta* growth coupled to a reduction in levels of defense metabolites, which already tended to be low, for asynchronously entrained pairs under constant dark (DD). We conclude that, while placing plants under constant conditions – especially DD – stresses plants and alters their capacity to mount resistance, there is no effect of in-phase versus out-of-phase entrainment of plants and herbivores on *N. attenuata*’s ability to resist *M. sexta* attack.

Results

Growth of *M. sexta* larvae

We entrained plants under one of two regimes: in-phase or out-of-phase, infested plants with *M. sexta* larvae which had been entrained under the in-phase regime, and measured larval growth under diurnal (LD) or constant conditions (LL, DD; n=26-45 larvae, Figure 1). Under all three conditions, growth of *M. sexta* larvae (Figure 2) was similar on in-phase and out-of-phase plants ($P > 0.13$, statistical test results in Table S1). 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 (DD, 27% mortality; LL, 43%; LD, 37%; DD vs. LL, $P = 0.038$; DD vs. LD, $P = 0.300$, Bonferroni-corrected G-tests of independence). Mortality did not differ between larvae on in- and out-of-phase plants (DD, 33.3 % and 20.0%, $P = 0.2870$; under LL, 48.9% and 37.8%, $P = 0.1510$; under LD, 41.7% and 31.7%, $P = 0.2734$). While larvae also developed through instars at similar rates on in-phase and out-of-phase plants, development through instars was slower under DD when compared to LL (day 9: DD, mode: end of 3rd to beginning of 4th instar; LL: middle to end of 4th instar).

Untargeted metabolomic fingerprints of in-phase and out-of-phase plants

Metabolomic fingerprints measured in positive electrospray mode, which captures the majority of known induced metabolites in *N. attenuata* (Gaquerel *et al.* 2010; Kim *et al.* 2011), were similar for in-phase and out-of-phase rosette-stage *N. attenuata* plants after 3 d of herbivory by *M. sexta* larvae under LL, DD, and LD (n = 5 plants). We used both principal component analysis (PCA, Figures 3, S1) and volcano plots (Figure S2) to visualize the differences between sample groups. Metabolomic fingerprints of in-phase and out-of-phase plants in PCAs differed before herbivory in LL and LD, but did not differ after 3 d of herbivory, as revealed by overlapping confidence intervals for the sample groups; similarly, overlap was greater in DD after 3 d of herbivory (Figure 3). The explanatory value of PCA axes for the separation of sample groups is difficult to compare between individual PCAs, but separation in metabolomics fingerprints 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.

Transfer to constant conditions (LL and DD) itself had a strong effect on plant metabolic fingerprints: adding plant samples taken before a 1 d pre-infestation period (see methods) to PCAs for LL and DD shows that in-phase (12 h light:12 h dark) and out-of-phase plants (12 h dark:12 h light) in fact became less similar after 1 d of exposure to constant conditions (Figure S1). Only a small number of m/z features (20) had a more than two-fold change and a P value < 0.05 in a comparison of in-phase and out-of-phase plants after 3 d herbivory in LL. Similarly, only 14 m/z features showed significant differences between in-phase and out-of-phase plants after 3 d herbivory in LD, while 66 m/z features showed significant differences between in-phase and out-of-phase plants after 3 d herbivory in DD (Figure S2). Hyponasty and strong reductions in starch contents were observed for plants in DD (Figure

S3), indicating that stress responses to DD might have modified the induced defense response.

Quantification of target secondary metabolites

Because separation along PC1 in PCAs (Figure 3) likely reflects changes to both primary and secondary metabolism induced by herbivory (e.g. Schwachtje and Baldwin 2008; Machado *et al.* 2013), we chose to quantify a number of well-characterized defense metabolites for which an effect on *M. sexta* growth had been shown in previous studies: nicotine (e.g. Steppuhn *et al.* 2004), 17-hydroxygeranylinalool diterpene glycosides (17-HGL-DTGs, Heiling *et al.* 2010), and caffeoylputrescine (CP, Kaur *et al.* 2010). Because we were interested in the effect of entrainment on the induced plant defense response, we show targeted metabolite data for the time point after 3 d herbivory ($n = 5$ plants, Figure 4). These defense metabolites accumulated to similar levels for in-phase and out-of-phase plants under LL and LD, except for CP under LL (Figure 4, $t = -2.649$, $df = 8$, $P = 0.0293$, all other P values > 0.17 , Table S2). Accumulation of CP was in fact lower for in-phase than out-of-phase LL plants, which is not consistent with the hypothesis that in-phase plants are better defended against herbivores.

However, in-phase plants accumulated significantly more nicotine and HGL-DTGs than out-of-phase plants under DD (Figure 4, nicotine: $t = 2.901$, $df = 8$, $P = 0.0199$; HGL-DTGs: $t = 2.327$, $df = 8$, $P = 0.0484$), consistent with the anticipatory defense hypothesis. In DD, levels of HGL-DTGs were very low in comparison to levels in plants grown under LL and LD conditions, and CP accumulation was too low to quantify. In fact, irrespective of entrainment, HGL-DTGs decreased over time in plants under DD (mean $\pm$ SE, $n = 5$, in relative peak area g^{-1} FM, before placement in DD: in-phase 66.4 ± 8.3 , out-of-phase 36.8 ± 6.3 ; after 24 h DD: in-phase 41.5 ± 9.1 , out-of-phase 20.6 ± 4.9 ; after 3 d herbivory [4 d DD]: in-phase 10.9 ± 2.4 , out-of-phase 4.6 ± 1.2); in contrast, nicotine accumulation under DD was similar to levels under LL and LD. These subtle differences in metabolites between in-phase and out-of-phase plants under DD did not affect *M. sexta* growth (Figure 2, Table S1). Rather, they highlight how constant conditions might dampen or alter defense induction, as does the fact that plants under LL accumulated HGL-DTGs to higher levels than plants under LD (Figure 4).

Feeding patterns of *M. sexta* larvae under diurnal and constant conditions

Previous studies have indicated a strong dependence of *M. sexta* activity on temperature (Kingsolver and Woods 1997; Petersen *et al.* 2000). Lamps in our climate chambers emit infrared as well as visible light, and we hypothesized that the infrared irradiation especially might have increased the temperature of chambers during light periods when compared to dark periods. In the *M. sexta* growth assays, 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 glasshouse, as well as in the field (e.g. Casey 1976), show that *M. sexta* larvae prefer to sit hidden under leaves. We thus measured temperatures below the surface of plants' leaves, at four different positions in each of the two climate chambers for at least 2 d, employing pendant data loggers. Temperatures during dark periods reflected the set temperatures of the

climate chambers (ca. 24 °C), but temperatures under leaves during light periods deviated from set temperatures by ca. 3 °C (Table S3).

We checked for diurnal fluctuation in *M. sexta* larval feeding activity over 24 h, following a 24 h acclimation period under DD, LL, and LD conditions and temperatures: 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). Larvae were fed on freshly cut *N. attenuata* rosette leaves from a common pool of plants under in-phase LD conditions. After 24 h of acclimation, leaf area removed by larvae over an additional 24 h in six 4 h periods was quantified as a measure of feeding activity (n = 5-6 larvae, Figures 5, S4).

Damage increased over the 24 h of the experiment under LL with a leveling-off toward the end of the experiment, which can likely be attributed to the initiation of molting by larvae (Figure 5a). Similarly, there was an increase in damage over the course of the experiment under DD without discernible fluctuations in inflicted damage (Figure 5b). Damage in leaf area removed was normalized by the initial larval mass which increased as larvae grew (Table S4), so the total amount of leaf area removed increased over 24 h, and the low slope of the increase in damage rates under the constant conditions highlights the strong interdependence of larval size and damage.

Under LD conditions, damage rates increased over the first three time intervals, but dropped in the 4th interval which followed the switch to the night regime, to increase again during the night (Figure 5c). However, in the 5th and 6th time intervals, in the middle and end of the night period, damage rates increased again. Damage rates measured under LD conditions in the 1st 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 intervals, between 13:00 and 21:00 (Figure S4, minimal linear mixed effects model, likelihood ratio: 26.36, $P < 0.0001$). In contrast, damage under LL and DD conditions occurred at a nearly constant rate per unit larval mass (Figure S4).

Thus, damage inflicted to leaves by *M. sexta* larvae rapidly and steadily increased with increasing caterpillar mass under all conditions, with evidence of diurnal fluctuation. Feeding damage reached the highest rates under LL and was lowest under DD conditions regardless of whether rates were corrected for larval mass (Figures 5, S4). This directly correlates with the differences in larval growth rates (Figure 2), changes to metabolic fingerprints under herbivory (Figure 3), and observed levels of HGL-DTGS and CP (Figure 4) under LD, LL, and DD.

Discussion

Goodspeed and colleagues (2012, 2013a,b) have proposed an “anticipatory defense” effect of enhanced plant resistance to insect herbivores conferred by correct entrainment with the environment and thus herbivore activity. This effect has so far been observed only with the generalist *Trichoplusia ni*, which displays circadian feeding activity on artificial diet (Goodspeed *et al.* 2012). Here, we show a counterexample, in which co-entrainment with the specialist herbivore *Manduca sexta* does not benefit the native tobacco *Nicotiana attenuata*.

Consistent with reports of physiological stresses experienced by plants under constant light or dark conditions (Graf *et al.* 2010; Sysoeva *et al.* 2010), our results also indicate that stress caused by constant experimental conditions may interfere with plant defense responses and thus confound results obtained under such artificial light and temperature regimes and thwart conclusions about the role of the clock in a plant's antiherbivore defense.

***N. attenuata* plants induced defense against *M. sexta* regardless of plant and larval entrainment**

Overall, *M. sexta* larval growth was similar on in-phase and out-of-phase *N. attenuata* plants under three different experimental set-ups (Figure 1) employing constant dark (DD), constant light (LL), or diurnal (LD) conditions, except for a small and transient, but significant increase in mass of larvae feeding on out-of-phase plants under DD. Larval growth was, however, slower under DD conditions, perhaps due to reduced feeding activity (Figure 5, Figure S4) or less nutritious plant material (Figure S3). Lower temperatures under DD, in the absence of warming infrared radiation from lamps, might have reduced larval growth rates (Figure 5, Table S3, Kingsolver and Woods 1997; Petersen *et al.* 2000). Metabolomic fingerprints of *N. attenuata* plants changed markedly after 3 d of feeding by *M. sexta* larvae under all three conditions, and there was little difference between in-phase and out-of-phase plants after herbivory, even though these groups differed prior to herbivory under LL and LD conditions (Figure 3). However, metabolomic fingerprints from plants in DD were generally less affected by herbivory than were those from plants in LL or LD (Figure 3). Accumulation patterns of HGL-DTGs and CP in particular (Figure 4) suggest that these compounds depend strongly on light and, likely, available carbon (Figure S3, Wünsche *et al.* 2011).

***M. sexta* feeding activity increases rapidly over time**

M. sexta larvae usually remain on plants from oviposition until they reach at least the third of five to six larval instars (e.g. Kingsolver 2007). The green larvae are mobile within their host plants, using them as camouflage, adopting postures similar to branches and hiding beneath leaves, which might also help them to thermoregulate in their native desert habitat (as shown for *M. quinquemaculata*; Kessler and Baldwin 2002b); and indeed the timing of feeding bouts in nature may be largely independent of temperature (Bernays and Woods 2000; Casey 1976) even though the rate of growth and development is not (Kingsolver and Nagle 2007; Kingsolver and Woods 1997; Petersen *et al.* 2000). The larvae, if they survive, may not leave the plant until there is little plant material left (Schittko *et al.* 2000).

Perhaps *N. attenuata* plants benefit most from inducing defense to the maximum possible level at maximum speed in response to *M. sexta* attack, anticipating the rapid growth of sedentary larvae rather than diurnal fluctuations in their activity. In this study, under all conditions, *M. sexta* damage rates increased by two- to five-fold within 24 h as larvae grew (Figure S4). This rapid increase in damage with increasing size of larvae might mask temperature- and daytime-specific fluctuations; for example, feeding rates of second-instar *M. sexta* under LD conditions transiently decreased at the beginning of the dark phase, and this pattern was more apparent when damage rates were normalized to increasing larval size (Figures 5, S4). This decrease was likely due to diurnal temperature fluctuations; when *M.*

sexta larvae were exposed to a 12 h:12 h LD regime at constant temperature, there was a fairly constant proportion of active animals over 24 h (Casey 1976). Differences in the magnitude of damage rates between the three settings (i.e. leaf area removed was greatest in the LL setting and was lowest in the DD setting; note different scales in Figure 5) are probably due to a combination of slightly different larval growth rates, and different incubation temperature, because increased temperatures elevate feeding rates and, consequently, increase developmental rates of *M. sexta* larvae (Kingsolver and Woods 1997, Petersen *et al.* 2000).

Typical temperature fluctuations in *N. attenuata*'s native environment of the Great Basin Desert in UT are much stronger than those in our climate chambers: in spring, when *M. sexta* eggs are laid on plants, temperatures may fluctuate by more than 20 °C over 24 h. It will be interesting to investigate whether this large temperature change results in predictable diurnal fluctuations in *M. sexta*'s feeding behavior in nature. If so, plants with a functioning circadian clock might in fact be able to anticipate diurnal rhythms of *M. sexta* feeding activity. The question is whether they would benefit from doing so, given *M. sexta*'s rapid growth and the likelihood that plants in nature are attacked by a varied community of other herbivores (see for example Glawe *et al.* 2003; Jander 2012; Schittko *et al.* 2000; Steppuhn *et al.* 2004; Steppuhn and Baldwin 2008).

When do plants anticipate attack, and when do they react to attackers?

In contrast to pathogen attack, which is strongly correlated to diurnal humidity and temperature cycles (Bhardwaj *et al.* 2011, Wang *et al.* 2011), herbivore activity may be less predictable on a daily time scale within a given environment. Rather, it is an intra- and interspecifically plastic trait subject to modification by external factors such as predator activity and availability of mating partners, and internal factors like nutritional status and hormones, in addition to the common circadian zeitgebers of light and temperature (Jander 2012; Wigglesworth 1972). In nature, a single plant is often infested by multiple herbivore species (Denno *et al.* 1995). Variation in climate and other environmental factors among habitats, and previous attack by particular herbivores, may be more reliable predictors of future herbivore attack than are plastic activity peaks, resulting in genetic and epigenetic adaptation of plant populations (Agrawal *et al.* 2012; Rasmann *et al.* 2012).

Nevertheless, some plant-herbivore pairs may be affected by circadian fluctuations in herbivore activity and plant defense capacity: *Arabidopsis thaliana* plants were more resistant to *T. ni* herbivores under both DD and LL conditions when plants and herbivores had been co-entrained (Goodspeed *et al.* 2012). In the case of *A. thaliana* and *T. ni*, it is difficult to interpret this interaction in an evolutionary context, since *T. ni* is a broad generalist, these two organisms have not co-evolved, and thus circadian fluctuations in *A. thaliana*'s defense response cannot be said to occur specifically in anticipation of *T. ni* feeding (Jander 2012).

One way to reconcile our data with those from Goodspeed and colleagues (2012, 2013b) is to hypothesize that these studies collectively describe a previously unrecognized difference between constitutive or generally damage-induced, and specifically herbivore-induced plant defenses (Bonaventure 2014). Constitutive production of plant defense metabolites may be,

at least in part, regulated by the circadian clock (Kerwin *et al.* 2011); and in the absence of reliable, specific, contraindicative information about attackers, plants may maintain these endogenous rhythms, as when the generalist *T. ni* attacks *A. thaliana* or harvested fruits and vegetables (Goodspeed *et al.* 2012, 2013b). However, if plants receive specific and reliable information about the identity of an attacker, they may be likely to engage in a targeted response which overrides constitutive rhythms.

A similar phenomenon has already been demonstrated for plant immunity: Wang *et al.* (2011) showed that *A. thaliana* plants rhythmically pulse the transcription of genes involved in basal and R-gene-mediated defense under the control of a core clock gene, the MYB transcription factor *CIRCADIAN CLOCK ASSOCIATED1* (*CCA1*); but following attack by the oomycete *Hyaloperonospora arabidopsidis*, the R-gene-mediated response disrupted the rhythmicity and increased the amplitude of *CCA1* gene expression, thus inducing sustained immune gene expression. The mechanisms behind the involvement of the clock in immunity have been further investigated by a handful of research groups using different strains of *H. arabidopsidis*, *P. syringae*, and pathogen-associated molecular patterns (PAMPs) (Bhardwaj *et al.* 2011; Wang *et al.* 2011; Zhang *et al.* 2013). A picture is emerging in which components of basal and R-gene-mediated defense fluctuate with individual circadian phases (expression of defense-related genes versus stomatal opening cycles), while specifically elicited responses may feed back on the clock to abolish constitutive rhythmicity – as has been shown for glucosinolate production in *A. thaliana* (Kerwin *et al.* 2011). This can be interpreted as an articulation of the long-known difference between constitutive and induced plant defenses, informed by an understanding of plant circadian clocks and their role in coordinating plant's activities (Harmer 2009; Kerwin *et al.* 2011; Kessler and Baldwin 2002a; Wang *et al.* 2011).

Interestingly, jasmonic acid (JA) and salicylic acid (SA) levels fluctuated constitutively in *A. thaliana* plants under DD and LL, in oppositely phased circadian cycles. Herbivory by *T. ni* increased the total amount of JA, but did not alter this rhythm, in contrast to the observations of altered rhythm following induction in plant-pathogen interactions (Wang *et al.* 2011; Zhang *et al.* 2013). The JA peak in wild-type (WT) plants preceded a maximum of feeding activity of *T. ni* towards the end of the subjective day and thus could have indicated anticipation of attack (Goodspeed *et al.* 2012). In accordance with this pattern of JA fluctuation, Shin and colleagues (2012) showed in *A. thaliana* that MYC2, a transcription factor which mediates jasmonate signaling, oscillates in transcript levels as well as protein levels throughout the day with a maximum toward dusk.

An alternative hypothesis is that the constitutive, circadian-controlled opposed oscillation of jasmonates and salicylates identified by Goodspeed and colleagues (2012, 2013a) is a general defense priming mechanism in *A. thaliana*, but not in *N. attenuata*. Many snail and slug species are known to attack *A. thaliana*, and these ravenous herbivores are both highly mobile, and generally nocturnal (Falk *et al.* 2013). Application of the locomotion mucus of at least one snail species to wounded *A. thaliana* leaves is known to induce accumulation of jasmonates (Falk *et al.* 2013), but many slugs and snails also induce salicylate-related pathways (Meldau *et al.* 2014), presumably via the pathogens that reside in their mucus. Attack by molluscs at night might mean a greater risk of infection with pathogens. As for

attack by pathogens, attack by molluscan herbivores is also highly dependent on predictable diurnal fluctuations in temperature and humidity, and the circadian clock is implicated in balancing pathogen defense with growth in *A. thaliana* (Zhang *et al.* 2013). Perhaps *A. thaliana* has evolved to anticipate more regular and predictable patterns of attack than the opportunistic, ephemeral desert annual *N. attenuata*.

Conclusions

N. attenuata supports a diverse herbivore community, among which the most damaging insect herbivore, *M. sexta*, may have an activity pattern dominated by rapidly increasing, rather than diurnally predictable activity. While defense responses to herbivores can reduce growth in *N. attenuata* (reviewed in Steppuhn and Baldwin 2008), plants which induce defense against herbivores also are fitter than those which do not (e.g. Zavala and Baldwin 2004), and thus evolutionary pressure may push *N. attenuata* plants not to anticipate attack, but to rapidly respond when it occurs. *N. attenuata* relies on constitutive as well as induced defense: for example, nicotine and 17-HGL-DTGs have both a constitutive and induced component (Baldwin 1999; Heiling *et al.* 2010); but the induced component dominates in magnitude and complexity (Wu and Baldwin 2010; Gaquerel *et al.* 2010), suggesting that constitutive fluctuations in *N. attenuata*'s defenses may be obviated by a strong induced response which might be less affected by the plant's internal rhythm. To the extent that there are diurnal fluctuations in *N. attenuata*'s induced defense metabolites (Kim *et al.* 2011), these may be indicative of physiological coordination between primary and secondary metabolism and might have little effect on plant resistance to herbivores. Studies employing transgenic *N. attenuata* plants with altered circadian clock components may allow a closer investigation of the relationship between plant and insect diurnal rhythms, in the natural conditions under which *N. attenuata* evolved.

Materials and Methods

Materials and methods for supplementary data, and expanded explanations of experimental and analysis procedures, are given in a supplementary materials and methods section.

Cultivation of *Nicotiana attenuata* plants

All experiments were performed with *N. attenuata* Torr. Ex. Wats. (Solanaceae) wild-type (WT) plants from a 31st-generation inbred line originating from a natural population at the Desert Inn Ranch in Utah, UT, USA in 1988 (Glawe *et al.* 2003). Germination and growth conditions until potting were as previously described (Krügel *et al.* 2002, Schuman *et al.* 2014). Twenty days after germination, individual small rosette plants were transplanted to 1 L plastic pots containing Frühsdorfer Nullerde (Hawita Gruppe, Vechta, Germany) with 0.5 g/L PG mix (Yara UK Limited, N E Lincolnshire, UK), 0.9 g/L Ca(H₂PO₄)₂, 0.35 g/L MgSO₄*7 H₂O (Sigma-Aldrich Chemie GmbH, Munich, Germany), and 0.055 g/L Micro Max (Everris GmbH, Nordhorn, Germany), and cultivated in one of two climate chambers (Johnson Controls Unitary Products, Norman, OK, USA; dimensions: 4.00 m l x 2.22 m w x 2.35 m h) under either an in-phase regime (light 09:00-21:00/dark 21:00-09:00) or an out-of-phase regime (dark 09:00-21:00/light 21:00-09:00) at 65% RH and 24 °C for at least another 10 d prior to the start of experiments. The 12 h light period reflects a typical photophase for

N. attenuata plants during vegetative growth and flowering (May-June), based on PAR measurements taken in the wash in Southwestern UT in which we conduct field research, which is part of *N. attenuata*'s native range (Y. Joo and colleagues, in preparation). Preliminary experiments demonstrated that *N. attenuata* plants flower under both long-day (16 h light/8 h dark) and 12 h light/12 h dark conditions. Light in climate chambers was provided by Master Sun-T PIA Agro 400 W (Koninklijke Philips N.V., Eindhoven, The Netherlands) at 100% intensity, ca. 400 $\mu\text{mol m}^{-2} \text{s}^{-1}$ PAR (measured using a LI-COR LI-250A Light Meter und Sensor LI-COR Quantum Q 35195, LI-COR, Lincoln, NB, USA). Plants were watered initially with 200 mL per plant, and from then on daily with 100 mL per plant. Peter's Professional Allrounder 20-20-20 N-P-K+TE fertilizer (Everris) was added once per week at a concentration of 0.75 g/L. Plants were used for the larval growth experiments under constant dark (DD), constant light (LL), or in-phase diurnal (LD) conditions after entrainment. To quantify larval feeding activity, leaves were cut every 4 h from a designated batch of rosette-stage plants cultivated under in-phase LD conditions, and batches were not re-used.

***Manduca sexta* larvae**

M. sexta eggs originated from an in-house colony at the Max Planck Institute for Chemical Ecology in Jena. Larvae hatched within 5 d after oviposition. For growth assays, eggs were incubated under the in-phase entrainment conditions in the climate chamber for at least 2 d, and for an additional 1 d under either LL or DD for experiments under constant conditions (Figure 1). Egg collection and incubation were timed such that most eggs hatched within the last 12 h of incubation. At the end of incubation, featherweight forceps were used to place freshly hatched *M. sexta* neonates on leaves.

***M. sexta* growth assays**

We decided not only to emulate the experimental set-up of Goodspeed and colleagues (2012) under constant conditions but, additionally, we established a set-up under diurnal conditions in which plants entrained under the out-of-phase regime (12 h:12 h dark: light, DL) were transferred to the in-phase regime (12 h:12 h LD) and kept under in-phase conditions for 2 to 3 d. A detailed justification is given in the supplementary materials and methods. Plants were entrained under either in-phase or out-of-phase conditions (see cultivation of *Nicotiana attenuata* plants) and *M. sexta* eggs were entrained under in-phase conditions (see *Manduca sexta* larvae). When *N. attenuata* plants enter the flowering stage, herbivory-induced defense responses are reduced (Diezel *et al.* 2011), but small rosettes would not have provided sufficient food for *M. sexta* larvae. We therefore used plants that were in full rosette to early elongating stages (31-33 d post-germination) at the start of assays. Larvae were kept under LL, DD, or in-phase LD conditions after entrainment until the end of the assay and transferred to new, entrained plants every 2-3 d to reduce habituation of plants to experimental conditions, or reduce the exacerbation of off-target effects due to prolonged exposure to constant conditions (Graf *et al.* 2010; Jänkänpää *et al.* 2012; Sysoeva *et al.* 2010). All plants were from the same synchronously germinated batch, so plants and larvae both aged over the course of the assay.

For diurnal (LD) conditions, at 9:00 on the first day of the experiment (lights-on in the in-phase chamber, lights-off in the out-of-phase chamber), 10 plants were transferred from the out-of-phase to the in-phase chamber. Six recently hatched *M. sexta* larvae were placed per plant on 10 in-phase and 10 out-of-phase *N. attenuata* plants (see Figure 1); larvae were distributed evenly among three similar, fully-developed rosette leaves on each plant. Plants of the same treatment were kept together in trays (5 plants/tray) distributed in the chamber, so that if larvae moved between plants, they were most likely to move onto a plant of the same treatment. After 3 d, 20 new out-of-phase plants were transferred to the in-phase chamber and plants in each treatment were exchanged for undamaged plants with the respective prior entrainment. Two to three larvae (if possible, from the same plant) were placed together on a new plant of the same treatment. Due to mortality, some larvae were lost and from day 4 some also moved between plants, and thus larvae, not plants, were treated as biological replicates. Subsequent exchanges of plants were conducted every 2 d as described. At the second exchange of plants (5 d) and at all remaining exchanges (7 and 9 d), larvae were weighed, since younger larvae experienced higher mortality after weighing; mass was determined to ± 0.001 g with a laboratory balance (Sartorius AG, Göttingen, Germany). Larval growth is interpreted as an indicator of both larval performance and plant resistance (e.g. Zavala and Baldwin 2004). The experiment was terminated when the largest larvae (4th or early 5th instar) began actively looking for host plants outside the trays containing plants of a single treatment.

Constant conditions were achieved using two climate chambers set to opposite 12 h:12 h LD cycles (the same chambers used simultaneously for in- and out-of-phase entrainment), so that plants for exchange remained entrained to the correct light regime, while organisms could be exposed to DD or LL by transferring plants and larvae between chambers every 12 h. For pre-infestation treatment and *M. sexta* infestation in DD, transfer without exposure to light was achieved by placing organisms in lightproof containers at the end of the 12 h dark phase and then transferring them to the other climate chamber after it entered the dark phase. Three green LED lights ($\lambda = 520$ nm, each 1 W,) 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 LL, organisms were transferred uncovered between chambers every 12 h. Experimental setups under DD and LL were conducted simultaneously. One day before *M. sexta* infestation, at 9:00 (lights-on in the in-phase and lights-off in the out-of-phase chamber), 5 each of in-phase and out-of-phase plants were transferred from their prior entrainment regime to the respective constant conditions. This 1 d pre-infestation exposure to constant conditions was done to avoid differences due to confounding effects from prior entrainment regimes (e.g. nutritive state; Goodspeed *et al.* 2012). Simultaneously, *M. sexta* eggs were also transferred to constant conditions. After 1 d under constant conditions, freshly hatched *M. sexta* larvae were placed on three fully developed rosette leaves of *N. attenuata* WT plants: 9 neonates/plant on 5 plants/treatment in each chamber; larvae were placed as described for diurnal conditions. After 3 d, plants in each treatment were exchanged for undamaged plants with the same entrainment and 1 d pre- infestation treatment in constant conditions. At the first exchange of plants (3 d), 10 plants of each treatment were used, so densities were reduced to 4-5 larvae/plant (if possible larvae were transferred from the same previous plant). In subsequent

exchanges of plants, we tried to even out the number of larvae per plant as number of larvae decreased due to mortality, and 3-4 larvae from one plant were placed together on a new plant. At the second exchange of plants and at all remaining exchanges, the larvae were weighed as described for LD. Again, the first exchange occurred 3 d after infestation while subsequent exchanges occurred at 2 d intervals (days 5, 7 and 9).

Plant tissue samples from *M. sexta* growth assays

For LD, one fully developed rosette leaf was collected from each plant before infestation with *M. sexta*, and after 3 d of herbivory, either the most heavily attacked, fully developed rosette leaf was collected, or a similar amount of leaf tissue was pooled from multiple such leaves, if these leaves all had similar amounts of tissue removed by *M. sexta*. For DD and LL, leaves at position 0 (oldest leaf undergoing the transition between sink and source) were collected before the 1 d pre-infestation treatment in constant conditions; leaves at position +1 (youngest source leaf) were collected prior to infestation with *M. sexta*, and leaves were collected as described for LD after 3 d of *M. sexta* infestation. The same plants were thus sampled 2-3 times. Midveins were removed, because small larvae normally do not feed on these leaf parts, and leaves were flash-frozen in liquid nitrogen and stored at -80 °C. Tissue was ground and aliquoted over liquid nitrogen immediately before extraction. Plants were treated as biological replicates for measures of plant metabolites.

Extraction of plant metabolites for fingerprinting and targeted analysis

Per tissue sample (n = 5 plants), 55 ± 5 mg of frozen, ground leaf material were weighed into 2 ml Eppendorf tubes and masses were recorded (± 0.0001 g weighed with a balance from Sartorius AG). Extraction of metabolites was performed as described by Gaquerel and colleagues (2010) using 500 μ L extraction buffer without internal standards (40% methanol [HPLC grade; VWR International Ltd., Leicestershire, UK], v/v, containing 40 mM acetic acid [Merck KGaA, Darmstadt, Germany] and 44 mM ammonium acetate [Sigma-Aldrich]; pH 4.8). Supernatants were transferred to a 1.5 mL HPLC crimP vial N11-1 HP with a 250 μ L micro-insert (Macherey-Nagel GmbH & Co. KG, Düren, Germany).

Metabolomic fingerprinting of plant tissue extracts

Plant extracts were analyzed by liquid chromatography-electrospray ionization-micro time of flight-mass spectrometry (LC-ESI-microToF-MS, Bruker Daltonik GmbH, Bremen, Germany) in positive mode as described by Gaquerel and colleagues (2010), but with specific modifications of the procedure as detailed by Kim and colleagues (2011) and with a 2 μ L injection volume. Raw data files from the Bruker software (Data Analysis v4.0) were exported as netCDF format and were processed as described by Kim and colleagues (2011), including peak detection, retention time correction, and annotation of isotope and adduct ions using the bioconductor XCMS and CAMERA packages (<http://www.bioconductor.org/>; Smith *et al.* 2006; Tautenhahn *et al.* 2008; Benton *et al.* 2010). XCMS output files were processed using Microsoft Excel 2010. We deleted all m/z features with a retention time 40 sec (calibration fluid), as well as features within the last 30 sec of the chromatogram (contaminants). We selected single abundant features for each compound by removing ions identified as low-abundance isotopes ([M+1], [M+2], etc.). Peaks were set to 0 if they had a relative intensity ≤ 90 or if their specific compound did not appear in $\frac{3}{4}$ of the replicates of

one treatment. Peak intensities of samples were normalized to g fresh mass. After processing, the data sets comprised 693 m/z features for LL, 604 m/z features for DD, and 654 m/z features for LD.

Quantification of target plant secondary metabolites

Plant extracts were also analyzed on a high-pressure liquid chromatograph coupled to a UV diode array detector (HPLC-DAD, Agilent Technologies, Santa Clara, CA, USA) as described by Oh and colleagues (2012). Peak areas were integrated using Chromeleon (version 6.8; Dionex Corporation, Sunnyvale, CA, USA) and the amount of metabolites in plant tissue was calculated by comparison with external standard curves of nicotine, chlorogenic acid (CGA), and rutin (Sigma-Aldrich; 7.8, 15.6, 31.3, 62.5, 125 or 250 µg/mL of each standard). To account for fluctuating measurement conditions, external standard curves were compared before, between, and after samples. Concentration values below the range of the standard curves were expressed as not quantifiable. Caffeoylputrescine (CP) was estimated based on external CGA calibration and expressed as µg CGA equivalents. HGL-DTG content was expressed as relative peak area. The amount of metabolites in the plant tissue was expressed as amount per g fresh mass.

Quantification of feeding activity

We quantified feeding activity as $\text{cm}^2 \cdot \text{g larval mass}^{-1} \cdot \text{h}^{-1}$ under the same light and temperature conditions used for *M. sexta* growth assays. Damage and larval mass were measured in 4 h intervals over 24 h, immediately following a 24 h acclimation period. We employed temperature/light data loggers to determine accurate temperatures experienced by *M. sexta* larvae during growth assays (HOBO UA-002-64 Pendant Temp/Light, Onset Computer Corporation, Cape Cod, MA, USA, accuracy: temperature: ± 0.53 °C from 0 °C to 50 °C; logging interval: 10 min; logging time: at least 2 d). Because *M. sexta* larvae are often found under leaves, we placed the loggers shaded under rosette leaves on soil of potted plants. Loggers were placed in four positions distributed throughout each of the two climate chambers used for *M. sexta* growth assays.

We 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; and for LD, 12 h light at 26 °C/12 h dark at 24 °C; humidity was set to 65% as in the climate chambers used for growth assays. Precautions to avoid light exposure for DD conditions and the dark phase of LD conditions were similar to the *M. sexta* growth assays: chambers were shielded from external light sources with curtains across the doors; in the dark, during work in the growth chamber, red light at a wavelength thought to be invisible to *M. sexta* larvae was used ($\lambda = 720$ nm; Briscoe and Chittka 2001) and for the collection of rosette leaves in the dark phase of the climate chamber, green LED lights were used as previously described.

Experiments began at 9:00, the time used to switch between light and dark periods in growth assays. *M. sexta* eggs were incubated for 3 d in an in-phase climate chamber (see *M. sexta* growth assays) and after hatching, 9-12 larvae/plant were placed on 5 plants and reared for 3-4 d until they reached the 2nd instar. The time was chosen such that caterpillars would not

molt during the 24 h of quantification. Five larvae were placed in a square Petri dish with plant material (n = 8 dishes) and acclimated under the light and temperature conditions of the respective assay 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. Immediately following the 24 h acclimation, the larvae in each Petri dish were weighed together (see Table S4) and provided with two fresh rosette leaves. After each 4 h interval, larvae were again weighed, old leaves were replaced with two freshly cut rosette leaves as described, and old leaves were scanned to quantify consumed leaf area. 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, Munich, Germany) 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., IBM, Armonk, NY, USA; in “fill” mode, with the following settings - fill: threshold method: auto threshold: % range: 25). Leaf area removed was normalized by dividing by the actual time between exchanges of 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. 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 %) and the corresponding replicates 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 began to molt; thus, the final number of replicates per assay was n = 5-6.

Statistical analyses

Statistical tests of differences in *M. sexta* growth, targeted defense metabolites, and *M. sexta* feeding damage rates were performed in R (version 2.12.2, 2.15.0, or 3.0.2) using RStudio (version 0.97.551) (R Core Team, 2012). Metabolomic fingerprinting data were analyzed in MetaboAnalyst (<http://www.metaboanalyst.ca/>, Xia *et al.* 2009; Xia and Wishart 2011): after first autoscaling to achieve normality and homoscedasticity, a principal component analysis (PCA) was conducted for all sample groups within a light regime (before transfer to constant conditions, before herbivory, and after 3 d herbivory) and, separately, only for the sample groups collected before and after 3 d herbivory within a light regime. Additionally, volcano plots were generated to filter for significant differences in the abundance of individual m/z features between leaf tissues of in-phase and out-of-phase plants after 3 d herbivory. Significant differences within an m/z feature were defined as a more than twofold change and a *P* value < 0.05.

For *M. sexta* growth and targeted metabolite data, we checked treatment groups graphically (quantile-quantile plots) and statistically (Shapiro-Wilk tests) for normality, and used F tests to check for homoscedasticity. 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 by log transformation, we conducted Wilcoxon rank-sum tests for independent samples.

In order to investigate whether the leaf area consumed by *M. sexta* larvae differed between 4 h time intervals, and thus probably also light and dark periods, linear mixed effects models (lme; nlme package, Pinheiro *et al.* 2013) were performed. We corrected for the fact that heavier larvae consume more plant material using the leaf area removed per g initial larval mass per h as response variable (Table S4). Explanatory variables were the respective 4 h time intervals (fixed effect), and the replicate ID was set as a random intercept. The optimal fixed structure was found by stepwise model simplification and factor level reduction followed by the comparison of the models using 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).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Experimental designs for assaying entrainment effects under constant or diurnal conditions.

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 panels. *Manduca sexta* larvae always received the in-phase entrainment regime while *Nicotiana attenuata* plants received in-phase or out-of-phase entrainment regimes. Angled arrows from above show time points at which leaf tissue 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. Plants were grown in their respective entrainment regimes for at least 10 d, while *M. sexta* were entrained as eggs and neonates for at least 1 d before infestation of plants (larvae

hatched within the last 12 h before infestation). Plants and eggs were transferred from their respective entrainment regime to constant conditions 1 d before infestation (a), or for diurnal conditions (b), out-of-phase plants were transferred to the light/dark regime under which in-phase plants and larvae had been entrained, and infestation followed immediately. *M. sexta* infestation lasted for 3 d, after which larvae were placed on new, entrained plants of the same treatment group. After day 3, transfer to new plants occurred every 2 d (not depicted).

Figure 2. *Manduca sexta* larvae grow similarly on in-phase and out-of-phase *Nicotiana attenuata* plants under constant or diurnal conditions.

Shown are means $\pm$ SE; for constant light (LL), $n = 23-29$; for constant dark (DD), $n = 30-37$; and for diurnal conditions (LD), $n = 35-46$ larvae. Treatment labels in panel (a) apply to all charts. *M. sexta* growth was monitored from day 5, to avoid increasing mortality of 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 Figure 1). (a) In LL, larval performance was similar on in-phase and out-of-phase plants for all three time points. (c) Also in LD, larval performance was similar at all three time points and larval had a similar final mass on day 9 as in LL. (b) In DD larvae on out-of-phase plants were significantly heavier (* - p -value < 0.05) on day 7, but not for the other two time points. Furthermore, final larval mass on day 9 was much lower in DD than in LL or in LD. Thus, larval growth did not differ overall on in-phase versus out-of-phase treatments under any of the three light conditions. Wilcoxon rank-sum tests were performed to compare treatments within each time point and regime.

Figure 3. 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 fingerprints of plant samples ($n = 5$ plants/treatment) taken before or after *M. sexta* infestation for each regime. Filled symbols stand for samples collected before herbivory, and empty symbols stand for samples collected after 3 d herbivory, under the respective light regime. Dashed and dotted lines delineate 95% confidence intervals; groups for which confidence intervals do not overlap are considered significantly different. Treatment groups differed due to induced defense in response to 3 d of herbivory (principal component 1, PC1) and effects due to prior

entrainment conditions (PC2) as well as by the effects of continued exposure to the respective light regime (PC1 and PC2), and interactions of these factors (see also PCAs in supplementary information for sample groups collected before pre- infestation treatment; similar effects explain the separation along PC1 and PC2 in those cases). (a) In LL, confidence intervals of treatment groups (in-phase and out-of-phase) did not overlap before herbivory, but did so after herbivory. (c) Similarly, in LD, confidence intervals overlapped after, but not before herbivory. (b) However, in DD, confidence intervals overlapped before and after herbivory, although the zone of overlap grew after herbivory. Overall, in-phase and out-of-phase plants became similar after 3 d herbivory under the respective light regimes.

Figure 4. Specific herbivory-induced defense metabolites in leaves of *Nicotiana attenuata* respond differently to entrainment as well as light regime.

Concentrations of nicotine (a), total hydroxygeranyllinalool diterpene glycosides (HGL-DTGs, b) and caffeoyl putrescine (CP, c) in in-phase or out-of-phase plants after 3 d of *M. sexta* infestation ($n = 5$ plants/treatment). To facilitate comparisons between different light regimes, panels within a compound are shown with the same scale, but not inset for HGL-DTGs in DD. *Nicotine and HGL-DTGs differed significantly ($P < 0.05$) between in-phase and out-of-phase plants under DD while CP differed significantly under LL; all in-phase and

out-of-phase pairs were compared using Student's t-tests, and no other significant differences were found.

Figure 5. Damage by *Manduca sexta* larvae feeding on leaves of *Nicotiana attenuata* increases under all regimes.

Damage to *N. attenuata* leaves caused by five 2nd-instar *M. sexta* larvae after 24 h acclimatization under the three experimental regimes used (see Figure 1), mean $\pm$ SE (n = 5-6). Note that Y-axes have different scales. Overall, damage from *M. sexta* larvae (consumed leaf area in cm² h⁻¹) increased two- to fourfold within 24 h under all three regimes (a-c). However under LD conditions, the amount of damage decreased initially after the onset of the dark phase (c). For choice of temperatures, see Results text and Table S3. Damage normalized to larval mass is shown in Figure S4; masses are given in Table S4.