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Fitness consequences of altering floral circadian oscillations for *Nicotiana attenuata*[FA]

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High-Impact Article

Abstract Ecological interactions between flowers and pollinators are all about timing. Flower opening/closing and scent emissions are largely synchronized with pollinator activity, and a circadian clock regulates these rhythms. However, whether the circadian clock increases a plant's reproductive success by regulating these floral rhythms remains untested. Flowers of *Nicotiana attenuata*, a wild tobacco, diurnally and rhythmically open, emit scent and move vertically through a 140° arc to interact with nocturnal hawkmoths. We tethered flowers to evaluate the importance of flower positions for *Manduca sexta*-mediated pollinations; flower position dramatically influenced pollination. We examined the pollination success of phase-shifted flowers, silenced in circadian clock genes, NaZTL, NaLHY, and NaTOC1, by RNAi. Circadian rhythms in *N. attenuata* flowers are responsible for altered seed set from outcrossed pollen.

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INTRODUCTION

In the eighteenth century, Carl Linnaeus noticed that many flowers open at specific times of the day, and designed a garden known as the "flower clock" in which flower behavior revealed the time of day (Somers 1999). These diurnal rhythms in flowers that include floral opening/closing and scent emissions, likely coevolved with the activity times of pollinators to maximize outcrossing (Fründ et al. 2011). The scent profiles of some flowers, such as *Petunia axillaris* and *P. parodii* are under the control of internal clocks and synchronize emissions with the active time of the flower's pollinators (Hoballah et al. 2005). In the wild tobacco, *Nicotiana attenuata*, floral scent emission, as well as nectar production depend on daily rhythms (Euler and Baldwin 1996; Kessler et al. 2012); both have been shown to be important mediators of pollinations and essential for maximizing this plant's fitness (Kessler et al. 2015). In the genus *Aquilegia*, flowers have evolved fixed orientations to match the particular active periods of their main pollinators and so radiated by floral isolation (Fulton and Hodges 1999; Hodges et al. 2004); in a dynamic way, *N. attenuata* flowers adjust their upward or downward orientations (Yon et al. 2016) in synchrony with the active periods of their main pollinators.

To examine the ecological relevance of the diurnal rhythms of flowers, it is essential to choose a model system that offers the possibility of physical and/or genetic manipulations of floral rhythms (Resco et al. 2009). Several studies conducted under normal light/dark or constant light conditions have produced results consistent with the notion that the internal circadian clock regulates diurnal rhythms in flowers (Sweeney 1963; Hoballah et al. 2005; Fenske et al. 2015; Yon et al. 2016). Components of the circadian clock have been identified in the model plant, *Arabidopsis thaliana* (Nagel and Kay 2012), and these clock components are highly conserved across many plant species (McClung 2013). The main oscillator is composed of two morning components, *LATE ELONGATED HYPOCOTYLE* (*LHY*) and *CIRCADIAN CLOCK ASSOCIATED1* (*CCA1*), and two evening components, *TIMING OF CAB EXPRESSION1* (*TOC1*) and

ZEITLUPE (*ZTL*). The clock functions through several negative feedback loops: LHY/CCA1 repress the accumulation of *TOC1* transcripts; TOC1 protein also represses transcript accumulation of *LHY/CCA*; and ZTL ubiquitinates TOC1 for degradation (Kim et al. 2007). Altering the expression of clock genes produces arrhythmic or dysrhythmic plants, which display many developmental (Adams and Carré 2011) and metabolic (Wang et al. 2011; Goodspeed et al. 2012) defects. However, most of the plant species used to unravel the genetic mechanisms of the clock are poor models for the study of flower-pollinator interactions due to their domestication histories or pollination systems.

The wild tobacco, *N. attenuata* inhabits the Great Basin Desert of the USA, produces opportunistically out-crossing self-compatible flowers (Bhattacharya and Baldwin 2012) that interact with different pollinators: nocturnal hawkmoths (e.g. *Manduca sexta* and *Manduca quinquemaculata*) and diurnal hummingbirds (e.g. *Archilochus alexandri*) (Kessler et al. 2010). *N. attenuata* flowers display numerous diurnal rhythms that likely influence outcrossing success. Most flowers open at night when they produce nectar and emit benzyl acetone (BA), the main floral volatile compound that attracts nocturnal hawkmoths (Kessler et al. 2008), and close again by the next morning. A relatively small proportion of flowers remain closed and scentless on their first night and partially open in their first morning with reduced BA emissions and fully open during the next night (Kessler et al. 2010). These morning-opening flowers (MOF), which require a burst of jasmonate signaling for their maturation, are produced in greater frequency on herbivore-attacked plants and are visited by diurnal pollinators (Kessler et al. 2010). Additionally, *N. attenuata* flowers move vertically during the day – flowers face downward during the midday and upward during the night (Video S1) (Yon et al. 2016). These floral rhythms, the vertical movement, scent emissions, opening, and nectar secretions repeat for the entire lifespan of the flower (2–3 d). So, why do *N. attenuata* flowers show rhythmic upward vertical movement? Both the type of flight and the construction of the proboscis of *M. sexta* moths (Sprayberry and Suver 2011), may constrain how readily the moth can access the nectar reward, and so we hypothesized that flower orientations affect the success of cross-pollinations mediated by *M. sexta*.

Previously, we identified the homologous genes of the core clock components, *Arabidopsis LHY*, *TOC1*, and *ZTL* in *N. attenuata* (Yon et al. 2012). To manipulate floral rhythms, we independently silenced these clock genes in *N. attenuata* by transformation with gene-specific inverted-repeat (ir) constructs and found that silencing the clock genes alters the plant's floral rhythms (Figure 1) (Yon et al. 2012, 2016). Here we investigate the consequences of these floral rhythms for plant fitness by using these "time-altered" plants, as well as mechanical modifications of the floral angle under glasshouse conditions. Outcrossed pollination mediated by nocturnal *M. sexta* hawkmoths was assessed by measuring capsule and seed production from antherectomized flowers, as an estimate of plant fitness. By physically constraining, or genetically altering the floral rhythms, we show that the circadian clock can establish a time-dependent pollination strategy that may help the plant to optimize outcrossing rates.

RESULTS

Nicotiana attenuata flowers maintain an approximately 40° upward orientation from horizontal during their development. In the morning of the first day of opening, flowers move downward to approximately 90° below horizontal; these flowers return to upright orientation before dusk, open and release floral scents from their corolla limbs (Figure 1). By the next morning, flowers face down again and close their corollas. This vertical movement of flowers is repeated for 2–3 days under long day (LD, 16 h light and 8 h dark) conditions, with diminishing amplitude on the third day (Yon et al. 2016). Similarly, the accumulation of nectar continues for 2 days in flowers of *N. attenuata*. The secretion of nectar occurs mainly during evening and night hours, and thus in the upright orientation of a flower's first 2 days; nectar volume decreases on the third day (Kessler 2012).

Hand-pollinated antherectomized flowers restrained at the three angles (+45°, 0°, −45°) all produced a full complement of capsules and seeds ($F = 0.87$, $P = 0.43$). However, when pollinated by naïve *M. sexta* moths, the antherectomized flowers tethered at 45° and 0° produced 13 (65%) and seven (35%) capsules, respectively, and no capsules and hence no seeds were produced when flowers were tethered at −45° (Figure 2B).

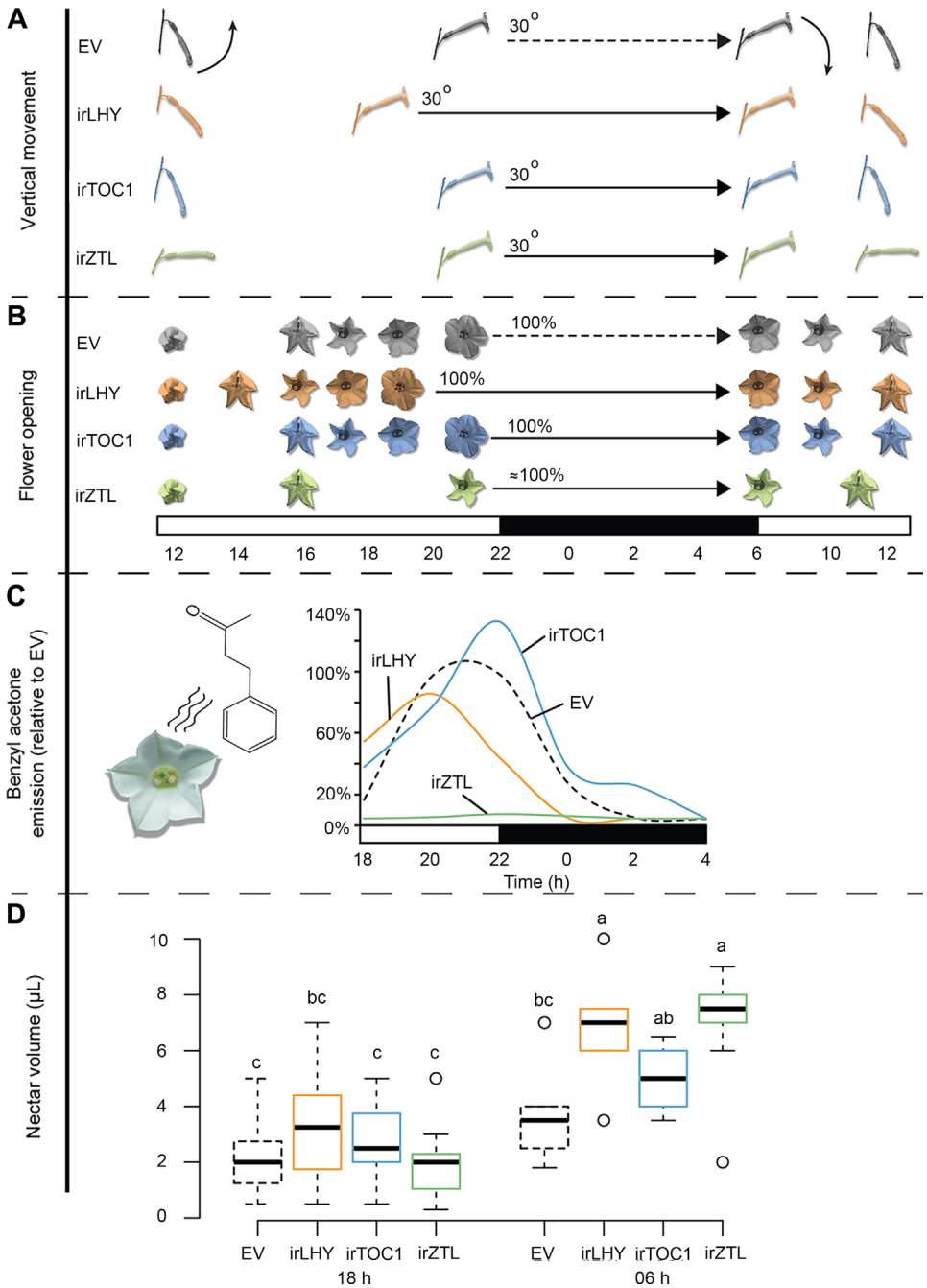

Figure 1. The circadian clock regulates floral rhythms in *Nicotiana attenuata*

Clock silenced genes: irLHY (LHY, LATE ELONGATED HYPOCOTYL); irTOC1 (TOC1, TIMING OF CAB EXPRESSION1); irZTL (ZTL, ZEITLUPE); and EV, control plant transformed with the empty-vector used to generate transgenic lines. Representations of **(A)** vertical movement and **(B)** aperture of the clock gene-silenced flowers on the first opening day. **(C)** Benzyl acetone (BA) emission trends in relative percentage to the maximum amount of BA emission from controls (*i.e.* EV flowers) from z-Nose^{TM} measurements. Inset figure depicts BA molecule. The data are the summary of previous research (Yon et al. 2016) and Figure S1. **(D)** Standing nectar volume in absence of pollinators, measured in microliters by pulse centrifugation extraction. All plants were grown under long day conditions (16 h light: 8 h dark). Each line is color coded: black-EV, orange-irLHY, blue-irTOC1 and green-irZTL.

Pearson's χ test for goodness-of-fit (hypothesis test described in Methods) and χ^2 test of independence between the flowers at 45° and 0° ($\chi^2 = 0.038$, d.f. = 2, $n = 20$, $P < 0.02$) indicated significant differences in hawkmoth-mediated cross-pollination success that depended on the angle of the flowers. The average number of seeds per capsule ($\pm SE$) was also strongly influenced: 144 ± 27 seeds in the flower at 45°, and 41 ± 18 seeds at 0° ($t = 3.49$, $P < 0.01$) (Figure 2A). Clearly, hawkmoths provide superior pollination services when flowers are at 45° and deliver more pollen to the stigma when flowers are oriented at 45° compared to 0° with respect to the horizontal.

With this experimental evidence of the importance of floral orientation, we used the clock gene-silenced plants to evaluate the ecological significance of floral rhythms in glasshouse assays that quantified outcrossing rates mediated by *M. sexta* pollinators. In previous research, we found that silencing circadian clock genes in *N. attenuata* alters diurnal rhythms in flowers; the flowers of irLHY plants opened, moved and released scent 2 h earlier compared to EV plants; flowers of irZTL plants partially opened, released no scent, and moved weakly (Figure 1). In contrast, irTOC1 flowers displayed all rhythms comparably to those of EV flowers (Figures 1, S1). Nectar in wild type plants is produced mainly between 6 p.m. and 4 a.m. (Kessler 2012). The nectar volume was not different among lines at 6 p.m., but differences were seen at 6 a.m. of the following day. The average nectar volume of irLHY (6.8 μL) and irZTL (6.9 μL) was larger and significantly different from EV (3.5 μL), but not from irTOC1 (5.0 μL) (Figure 1D); the nectar volumes of EV and irTOC1 flowers did not differ.

The differences between EV and irLHY, as well as irZTL plants are likely associated with the different oscillation times of these plants. Flowers of irLHY plants move 2 h earlier to an upright position than EV flowers,

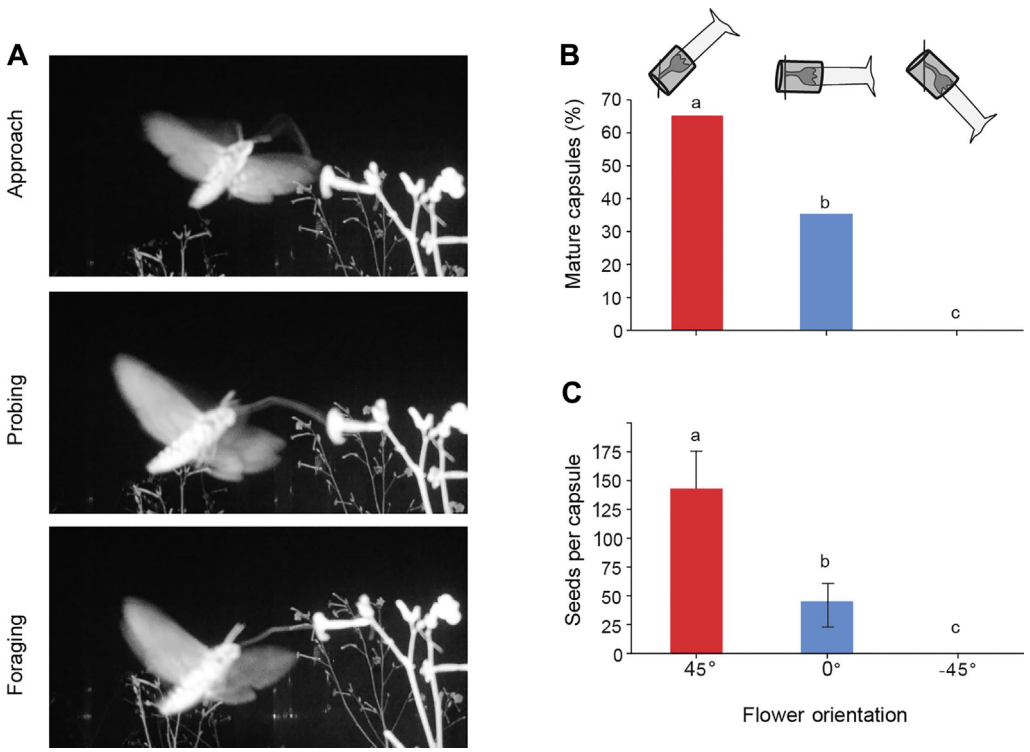

Figure 2. Flower angles determine rates of outcrossing mediated by *Manduca sexta* moths
(A) *M. sexta* approaching, probing and foraging on *N. attenuata* EV flower facing naturally upwards. Photo sequence taken at approximately 22 h with a wild-camera Snapshot Mini (Dörr, Germany), equipped with a PIR sensor camera and IR flash. Percentages of **(B)** mature capsules and **(C)** mean ($\pm SE$) number of seeds per capsule in emasculated WT flowers which were fixed at each of three positions (−45°, 0°, 45°). Different letters indicate significant differences among the flower positions as determined by Pearson's χ^2 test ($P < 0.05$) for capsule formation and one-way ANOVA followed by Tukey *post-hoc* test ($P < 0.01$) for seeds produced per capsule.

and accumulate nectar earlier than do EV flowers, as seen in the nectar volume trend at 6 p.m. The irZTL flowers have the larger nectar volume in the morning, which does not significantly differ from that of irLHY flowers.

We used both single (no competition) and paired (competition with EV) experimental designs to evaluate how *Manduca* could provide cross-pollination services to the different clock gene-silenced lines (Figure 3). For the no-competition experiment using a single line, we antherectomized a total of 25 flowers (five flowers/plant) on EV, irLHY, irTOC1, or irZTL plants and placed plants of a single line on the table with two naïve *M. sexta* moths and 10 WT pollen-donor plants for one night (Figure 3A). After 2 weeks, 60% of EV flowers had matured capsules, and similar pollination rates were observed in irLHY (60%) and irTOC1 (56%). However, irZTL (44%) tended to produce fewer capsules than did flowers from EV plants. Seed numbers per capsule did not differ among the lines (Figure 3A).

In contrast, when clock gene-silenced plants competed with EV plants for the *Manduca*-mediated pollination services, out-crossing pollination success differed significantly. irZTL plants produced half the number of capsules than did EV plants (Figure 3B, $t = 3.1$, d.f. $= 4.4$, $P < 0.05$) and this difference in capsule number was larger than the difference found between EV and irZTL plants when pollinated singly (Figure 3A, B). EV plants in the EV-irZTL competition

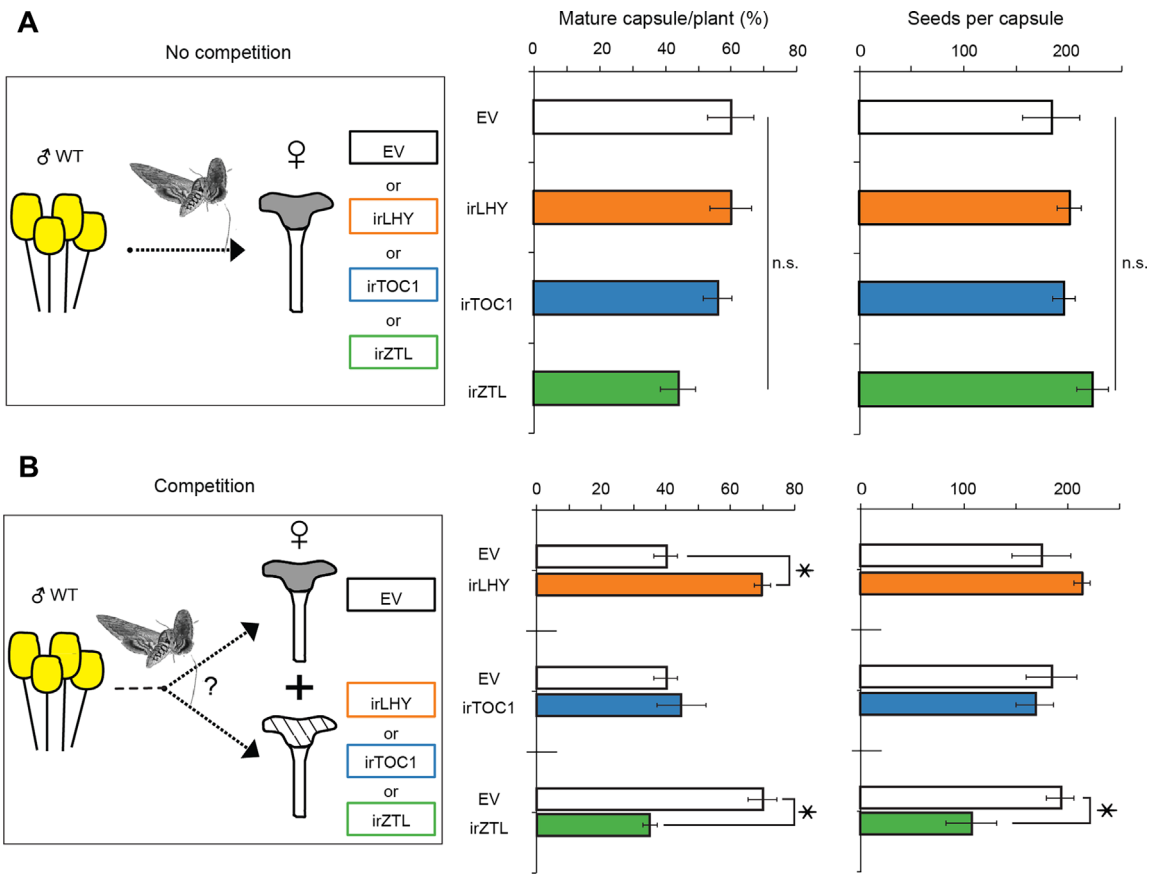

Figure 3. The circadian clock coordinates pollination success in *N. attenuata*

(**A**) Flowers of EV, irLHY, irTOC1, and irZTL plants were emasculated and individually exposed to two *M. sexta* moths for one night with 10 WT pollen donor plants (no competition design). (**B**) In the competition experiments, each of the clock gene-silenced lines were individually paired with EV plants and their flowers competed for the pollination services of two *M. sexta* moths which also had access to 10 WT plants as pollen donors. Mean (± *SE*) percentage of mature capsules per plant and mean (± *SE*) number of seeds per capsule resulting from pollinating emasculated flowers by *M. sexta* moths. Asterisks represent significant difference between EV and clock gene-silenced lines (** $= P < 0.01$, Student's *t*-test).

pairs produced 75% more capsules than in other experimental pairs. In addition, the irZTL line produced significantly fewer seeds per capsule ($t = 4.01$, d.f. $= 9.6$, $P < 0.05$) than the EV pair (Figure 3B). EV-irTOC1 pairs did not differ either in capsule formation or in number of seeds per capsule (Figure 3B). Unexpectedly, in the EV-irLHY pairs, irLHY plants produced almost twice as many capsules ($t = 3$, d.f. $= 5.6$, $P < 0.05$) as did EV, an outcome opposite of the results of the EV-irZTL pairs (Figure 3B). There was no significant difference in the numbers of seeds per capsule in the EV-irLHY pairs ($t = 1.34$, d.f. $= 8.1$, $P = 0.21$).

DISCUSSION

Ecological consequences of flower orientation

Several hypotheses have been proposed about the role of flower orientation in outcrossing success. For example, the horizontal or downward orientations of flowers increase pollen transfer because pollinators are in contact with flowers for a longer time due to an increase in handling time to obtain the reward (Fenster et al. 2009). In contrast, the upward orientation of flowers could facilitate visual recognition from multiple directions by pollinators despite a reduction in pollen transfer (Ushimaru and Hyodo 2005; Fenster et al. 2009). Here we show that the upward orientation of *N. attenuata* flowers at night improves the outcrossing success mediated by naïve *M. sexta* moths, allowing an easier access to the nectar at the bottom of the flower given the stiffness of the moth's proboscis. The downward facing flowers produced no capsules, and this result is consistent with those of studies with *Hyles lineata* and *Aquilegea* plants, in which pendent flowers were rarely visited by hawkmoths (Fulton and Hodges 1999). This vertical movement of flowers might be a particular adaptation of *N. attenuata*, as closely related *Nicotiana* species are not known to move their flowers rhythmically downward and at most only bend them upwards. These results suggest that the plant's circadian clock entrains flower orientation to facilitate the recognition, probing, and foraging of *M. sexta* (Figure 2), and hence has a different function than that proposed for the clock in stem circumnutation and leaf movement (Stolarz 2009).

Downward orientation of flowers can provide several advantages: It reduces susceptibility to florivores (Ashman and Schoen 1994) and nectar desiccation caused by solar radiation (Kessler 2012), and it could filter out the visits of diurnal pollinators (Fenster et al. 2004). Nectar accumulation in *N. attenuata* flowers decreases, while the sugar concentrations increase during the day, presumably as a consequence of the strong solar irradiance in the Great Basin Desert (Kessler and Baldwin 2007; Kessler et al. 2012), even though flowers face downward and close during the day. If flowers would face upward and remained open during the day, this nectar concentration effect could be much greater.

However, the vertical movement is not likely to protect flowers from nectar robbery by carpenter bees, which collect nectar by puncturing the corolla tube base at dawn and dusk (Kessler et al. 2008); the upward orientation is attained before nightfall when the robbers are active, suggesting that flower movement alone would not prevent damage by these opportunistic nectar robbers. However, the downward orientation of the corolla may reduce its visibility to robbers or florivores.

Ecological implication of the circadian clock

A fundamental assumption in chronobiology is that the circadian clock increases the fitness of organisms. Therefore, we predicted that dysrhythmic/arrhythmic traits in the clock-altered flowers would reduce their out-crossing pollination success. This is what was observed for the flowers of irZTL plants but not for the flowers of irLHY plants (Figure 3B).

As expected, cross-pollination rates in irZTL plants were reduced when irZTL plants competed with EV plants for the pollination services of naïve *M. sexta* moths (Figure 3B). However, irLHY flowers had higher night pollination rates when they competed with EV flowers (Figure 3B). At first, the clear advantage of irLHY flowers in competing for the *Manduca*'s pollination services appears to be consistent with their earlier moving, opening, and scent-emission phenotype, but given that *M. sexta* moths were most active much later in the evening (10–11 p.m.), this is not likely the correct explanation as at this later time the flower angles, opening, and scent emissions were similar to those of EV flowers, providing no particular time advantage to be visited first. Similarly, the small increase in nectar

volume of irLHY flowers at 6 p.m. is not likely to explain its greater success in the trials. An alternative hypothesis is that other unmeasured floral traits, such as minor floral scent compounds, or UV floral pigments, may be altered in irLHY flowers. We predict that early advertisements in nature may decrease pollination success and increase the visitation of unfavorable insects, such as florivores, suboptimal pollinators or nectar robbers (Kessler and Baldwin 2011).

We expect that foraging moths will learn to associate particular flower traits with a nectar reward, but as we used naïve moths for all experiments, this associational learning would be expected to occur during the experiment itself and this is reflected in our results. To avoid having the results confounded by prior associations, non-competition experiments with single lines tested at a given time were used in all experiments reported here.

Circadian rhythms in flowers have evolved in response to interactions with mutualists and antagonists and also to synchronize with the environmental rhythms in their native habitats. Their main function is presumably to ensure outcrossing services by attracting pollinators at the right time (Jones and Little 1983; Harder and Barrett 2006). *N. attenuata* is an interesting model species with which to study the function of floral rhythms for plant-pollinator interactions, because its three floral rhythms —aperture, vertical movement, and BA emission — can function to attract different types of pollinators (Kessler et al. 2010). The mechanical and genetic manipulation of floral rhythms clearly reveals that altering circadian rhythms in flowers affects pollination success.

It is thought that the circadian clock helps plants to anticipate abiotic factors, such as preparing the photosynthetic machinery at dawn to anticipate the rising of the sun (Green et al. 2002) or to increase its pollination service (Vandenbrink et al. 2014). The clock has been argued to help plants anticipate attack from herbivores (Bhardwaj et al. 2011; Wang et al. 2011; Goodspeed et al. 2012; Zhang et al. 2013), an inference which has not found support in the *N. attenuata* system (Herden et al. 2016). Based on evolutionary considerations, beneficial biotic interactions, rather than antagonistic interactions, are more likely to be usefully anticipated by a circadian clock, because antagonists can readily counter a plant's clock-mediated anticipation by changing the timing of their attack. The evidence provided here, demonstrates that *N. attenuata*'s clock exquisitely prepares its flower for pollination by *M. sexta* moths and in doing so, exhibits a botanical version of synchronized dancing.

MATERIAL AND METHODS

Plant growth conditions

We used *Nicotiana attenuata* Torr. Ex. Wats (Solanaceae) plants (30th inbred generation) and isogenic transformed plants that all originated from the same accession in Utah. Seeds were sterilized and germinated on Petri dishes and grown under long-day conditions (LD, 16 h light/ 8 h dark) in a growth chamber for 10 d until being transferred to 1 L pots in a glasshouse located in Jena, Germany, as described in Krügel et al. (2002).

Floral traits of the clock-gene silenced lines in *N. attenuata*

NaLHY (NCBI accession number JQ424913), NaTOC1 (JQ424914) and NaZTL (JQ424912) were independently silenced by transformations with gene-specific inverted repeat (ir) constructs driven by the CaMV 35S promoter (Yon et al. 2012, 2016). Two independently transformed T_2 and T_3 homozygous lines (irLHY-404, irLHY-406, irTOC1-205, irTOC1-212, irZTL-314, and irZTL-318) were used to characterize the diurnal rhythms in flowers (Yon et al. 2016) and all pollination experiments described in this study were conducted with the fully characterized irLHY-406, irTOC1-205, and irZTL-314 lines (Yon et al. 2016). Empty-vector (EV) plants were used as controls for the silenced lines in the pollination experiments, and the 31st inbred generation wild-type (WT) plants from the same accession from Utah were used as pollen donors. The floral traits of the clock-altered lines used in this study are summarized in Figure 1A–C from data previously published (Yon et al. 2016), except for irTOC1 for which the primary data is presented in Figure S1 and acquired as described in Yon et al. (2016).

Benzyl acetone emission from the first opening flowers of irTOC1 was measured in real time using a portable gas chromatograph, z-Nose^{TM} 4200 (Electronic Sensor Technology, Newbury Park, CA, USA). To trap the headspace volatiles released from individual flowers, 50 mL plastic tubes (Falcon Plastics, Oxnard, CA, USA) were cut in half, and the upper parts with a cap were used, with a headspace volume of approximately

9,000 mm^3. A single hole was made in a cap through which the sampling needle of the z-Nose sampled the headspace of flowers.

Nectar volume was measured by collecting flowers at 6 p.m. of the first opening day and at 6 a.m. of the following day, and placing them in ice-cold Eppendorf tubes (0.5 mL) for a short centrifugation of approximate 2 s. Nectar was removed from the Eppendorf tubes and quantified with a calibrated pipette tip with 0.5 μL calibrations.

Cross pollination experiments

To measure cross pollination rates in the clock gene-silenced lines, stem vertical supported plants with antherectomized flowers were transferred to a table covered with a green mesh tent (1.8 m height × 1.6 m width × 6 m length) in a glasshouse cabin. Fully-developed flowers from LD-grown plants were emasculated in the early morning to prevent self-pollination. We chose experimental days when there were no other flowering *N. attenuata* plants in the glasshouse cabin. We manipulated the flower orientation by fixing them mechanically at three different angles: +45°, 0° and −45°, with soft plastic straws surrounding the flower pedicels and wire. Flowers move through a 140° arc each day and the three experimental angles represent the two extreme and the middle positions of this arc. Three emasculated flowers on 10 WT plants were fixed at each experimental angle, and 20 WT plants were placed as pollen donors. Three male naïve *M. sexta* moths were released at 20 h, to function as the sole pollinators for one night. On the next morning, the *M. sexta* moths and flower tethers were removed and the experimental plants remained on another table in the glasshouse to mature seed capsules in case of successful outcrossing; flowers that received no outcrossing aborted their flowers during this time. After capsule maturation, the number of capsules and seeds per capsule were counted. The experiment was repeated twice with the same number of emasculated plants and pollen donor plants. To control for possible fertility differences due to angle, a hand-pollination conducted under the same experimental conditions served as a control for the experimental manipulations. The small nectar volume of flowers (Figure 1D) and the narrow corolla tube prevents nectar from draining from flowers at any angle.

To measure outcrossing rates in the clock gene-silenced lines, plants with antherectomized flowers were transferred to the same table with a tent cover as described above. The experiment was conducted twice: once in which EV and all clock gene-silenced lines competed with EV plants for the pollination services of two naïve moths and once when each line was evaluated separately. Possible *M. sexta* learning effects can be excluded in these experiments, given that naïve moths were used, and these moths were exposed to only one line of flowers. In this way, the experiments avoided any confounding effects of a moth's previous experience. For the no-competition experiment, five flowers on five plants per each line (EV, irLHY, irTOC1, and irZTL) were antherectomized in the morning and provided with the pollination services of two naïve *M. sexta* moths, for one night in separate experiments. Moths obtained pollen while nectaring on 10 WT plants which were placed in the arena in parallel to the target pollen receiver plants, and thus served as pollen donors. For the paired-competition experiments, five flowers on four EV plants and five flowers on four plants of each of the clock gene-silenced lines (irLHY, irTOC1, and irZTL) in separate paired-experiments were emasculated in the morning while all other flowers were removed. These plants competed for the pollination services of two naïve *M. sexta* moths for one night. Again 10 WT plants placed in addition to the competing transgenic lines into the experimental arena, served as pollen donors. Each of the three clock gene-silenced lines competed against EV plants in separate experiments. EV and clock gene-silenced plants were arranged in pairs, with 30 cm distance between plants. The number of matured capsules and seeds were counted after ripening.

Statistical analysis

To evaluate the results from the flower angle manipulations on the discrete seed capsule formation, three different null hypothesis models were tested with a Pearson's χ test for goodness-of-fit: the first hypothesis was that cross-pollination by the hawkmoth does not differ among the three positions; the second was that pollination at flowers fixed at 45° and 0° angles was not different; the third was that flowers fixed at 45° are preferentially pollinated. The first and second null hypotheses were rejected ($P < 0.001$ and $P < 0.01$, respectively), and the third hypothesis (preference at 45°) was not rejected ($P > 0.3$). χ^2 test of independence was used between the flowers at 45° and 0°. The results from capsule number and seed number were

statistically analyzed using Student's *t*-tests and one-way ANOVAs, all were performed with R 2.15.3 (http://www.r-project.org/). Plants were used as replicates; in other words, matured capsules from a plant were averaged, and this plant average was used as the replicate, to avoid pseudo-replication. Nectar volumes from both time points were analyzed via generalized linear model, using time and transgenic line as factors with interaction terms. Each line per time was tested using *post-hoc* HSD. All tests were performed in R, the *post-hoc* HSD test was performed using the agricolae v.1.2-3 package.

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AUTHOR CONTRIBUTIONS

F.Y., S.K., and D.K. designed and performed experiments and wrote the manuscript. L.C.L. and Y.J. performed experiments. F.Y. and S.K. screened and characterized the transgenic lines. I.T.B. and S.K. conceived the study, coordinated and wrote the manuscript. All authors declare that they have no conflicts of interest.

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SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article: http://onlinelibrary.wiley.com/doi/10.1111/jipb.12511/suppinfo

Video S1. Vertical movement and opening of the *Nicotiana attenuata* flower

Figure S1. Effect of silencing TOC1 on *Nicotiana attenuata* floral traits in comparison to *empty-vector* (EV)

(**A**) Mean (± *SE*) flower angles. (**B**) Mean (± *SE*) distance between petal junctions on corolla limbs. (**C**) Mean (± *SE*) levels of BA emission measured using a z-Nose™ portable GC and BA concentrations were calculated using a standard curve of BA dilutions. Plants grown under 16 h : 8 h, light : dark (LD) conditions.

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