


Flower movement balances pollinator needs and pollen protection

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Abstract. Flower signaling and orientation are key characteristics that determine a flower's pollinator guild. However, many flowers actively move during their daily cycle, changing both their detectability and accessibility to pollinators. The flowers of the wild tobacco *Nicotiana attenuata* orientate their corolla upward at sunset and downward after sunrise. Here, we investigated the effect of different flower orientations on a major pollinator of *N. attenuata*, the hawkmoth *Manduca sexta*. We found that although flower orientation influenced the flight altitude of the moth in respect to the flower, it did not alter the moth's final flower choice. These behavioral observations were consistent with the finding that orientation did not systematically change the spatial distribution of floral volatiles, which are major attractants for the moths. Moreover, hawkmoths invested the same amount of time into probing flowers at different orientations, even though they were only able to feed and gather pollen from horizontally and upward-oriented flowers, but not from downward-facing flowers. The orientation of the flower was hence crucial for a successful interaction between *N. attenuata* and its hawkmoth pollinator. Additionally, we also investigated potential adverse effects of exposing flowers at different orientations to natural daylight levels, finding that anther temperature of upward-oriented flowers was more than 7°C higher than for downward-oriented flowers. This increase in temperature likely caused the significantly reduced germination success that was observed for pollen grains from upward-oriented flowers in comparison to those of downward and horizontally oriented flowers. These results highlight the importance of flower reorientation to balance pollen protection and a successful interaction of the plant with its insect pollinators by maintaining the association between flower volatiles and flower accessibility to the pollinator.

Key words: flower handling; flower orientation; *Manduca*; *Nicotiana*; pollen viability; pollination.

INTRODUCTION

Detecting and subsequently being able to handle a certain flower are two key steps in the interaction between pollinators and plants (Harder 1985, Riffell et al. 2014). However, the detectability and accessibility of a flower for a pollinator might vary, as some flowers actively open and close or alter their orientation during the course of the day (Atamian et al. 2016). It has been argued that flowers perform these movements to balance pollinator attraction and the protection of nectar and pollen (Aizen 2003, Yon et al. 2017). However, in spite of its potential importance for both the plant and the pollinator, experimental evidence for the effects of flower

orientation on pollinator behavior and flower protection has remained limited (Fenster et al. 2009).

For animal-pollinated plants, flowers are costly organs that encourage the receipt and export of pollen by attracting animal visitors that move between different plant individuals to ensure outcrossing. Hence, selective pressure has likely altered floral displays toward attracting suitable pollinators in such a way that visitation and pollen transfer are maximized while little investment is lost to unnecessary or detrimental floral traits. Rich odor bouquets, for example, might not be as important for attracting hummingbirds as for attracting hawkmoths, while investing in a long nectar spur is essential for ensuring pollen transfer by both pollinator groups (Fulton and Hodges 1999, Campos et al. 2015). Additionally, flower characteristics might also differ in their efficiency for attracting pollinators i.e., altering the corolla size might be more costly and thus less efficient than changing the corolla orientation, although both could increase pollination (Fenster et al. 2004, Ushimaru and Hyodo 2005).

However, plants will not only have to be efficient in their investment in flower features with regard to their effectiveness in attracting pollinators, but also with regard

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to costs arising through potential dangers caused by these flower characteristics. Large and dark-colored corollas, for example, certainly help to attract hummingbirds (Schiestl and Johnson 2013), but also increase water loss through higher evapotranspiration (von Arx et al. 2012). Similarly, floral features increasing hawkmoth pollination might also increase the risk of the plant to be attacked by the offspring of these pollinators (Kessler et al. 2010). Ultimately, it might therefore be expected that flowers invest in traits that increase pollination in a way that is cost effective but also balances potential fitness gains against abiotic and biotic risks.

The orientation at which a flower faces the approaching pollinator is an important trait that alters the way a pollinator detects and perceives a flower. Some nectar-feeding bats, for example, locate flowers by the acoustic reflection of a specifically shaped corolla petal. The strength of the echo-reflection strongly depends on the orientation of the flower petal, making flowers with downward facing petals nearly undetectable to pollinating bats (von Helversen and von Helversen 1999). Similarly, orientation might influence the visual detectability of a flower to a certain pollinator by altering its display size and could thereby result in an ethological isolation between plant populations with natural variations in the orientation of their flowers (Fulton and Hodges 1999). In contrast to sound and sight, the olfactory signal of a flower does not travel linearly. Instead, directly after their release floral volatiles are altered by air movement, which is, in part, determined by the vortex created by the flower in windy environments (Ortega-Jimenez et al. 2013, Riffell et al. 2014; for review, see Riffell et al. 2008). Therefore, the orientation of the corolla might not be reliably reflected in the volatile signals of a flower. Changes in flower orientation that are inconsistent with the flower's volatile signals would thus have particularly negative effects on pollinators that rely strongly on olfaction during foraging as they would be unable to detect flowers that are not accessible to them due to their altered orientation. These links between flower orientation and flower signals might thus require a close synchronization of these two traits in order to ensure successful pollination.

In addition to flower detectability, flower orientation also alters the time and energy that a pollinator needs to invest in order to access nectar and pollen (Harder 1985). Pollen collecting bumblebees for example might profit from flowers oriented downward, as pollen falls more easily onto their body (Heinrich 1979). Hence, less pollen might be collected by bees visiting upward-oriented flowers, forming a potential mechanical barrier for a successful pollen transfer between plants with flowers oriented at different angles. Conversely, syrphid flies and nectar collecting bumblebees land less frequently on flowers with downward orientations, although they approach flowers of all orientations equally often (Ushimaru and Hyodo 2005). The interplay between these ethological and mechanical isolation barriers is not well

understood as it remains often unclear to what extent pollinators perceive and respond to the accessibility of a flower (Campbell et al. 2016).

In addition to this, flower orientation has also been proposed to prevent nectar dilution and protect pollen from rain in high precipitation areas (Aizen 2003), while it can also be presumed that a downward orientation can protect the flower from high temperatures in arid habitats. High temperatures and low relative humidity levels can severely decrease or alter the nectar sugar concentration, and thereby strongly affect the suitability of the nectar to certain pollinators and thus alter their flower choice (Nicolson 1998, Contreras et al. 2013). In addition, heat stress might also affect pollen viability and thereby directly reduce plant fitness (Zinn et al. 2010). Plants in arid environments might hence be under additional selection pressure to combine or to balance flower orientation for pollinator attraction and flower protection.

Nicotiana attenuata is a native plant of the Great Basin Desert (USA) where it is pollinated by *Manduca sexta* and other pollinators (Fig. 1a; Kessler et al. 2008). *N. attenuata* displays a strong circadian rhythm, in which the flowers move daily to a horizontal or upward orientation during sunset and to a downward orientation after sunrise for the 3 d of their flowering time. Hence, the flower movement only takes place during short transition periods and the flower then remains at a certain orientation for most of its daily cycle (Yon et al. 2016). Parallel to this, floral volatile emissions are most pronounced during the first half of the night and largely absent during daytime (Yon et al. 2016). These volatile emissions have been shown to be of particular importance for the interaction between *N. attenuata* and its night-active pollinator, the hawkmoth *M. sexta*. Moths detect floral emissions with their antennae (Riffell et al. 2013, Haverkamp et al. 2016a) as well as with specific sensilla on their proboscis (Haverkamp et al. 2016b). However, although these effects of floral volatile emissions have been intensively studied, other floral characteristics such as flower orientation might also have a strong influence on the interaction between *N. attenuata* and its pollinators.

Here we tested how floral orientation affects plant-pollinator interactions of *N. attenuata* and *M. sexta*, by experimentally altering the angular position of flowers in wind tunnel assays. We found that these alterations in the flower orientation strongly influenced the behavior and the foraging success of the moth as well as the pollen uptake and delivery for the plant. Furthermore, we assessed how flower orientation influences the temperature in the vicinity of the anthers and show that flowers facing upward during day time have reduced pollen viability. Active flower reorientation thus enhanced pollination service as well as the protection of the reproductive tissue and thereby likely repays for any resources invested by the plant into this flower movement.

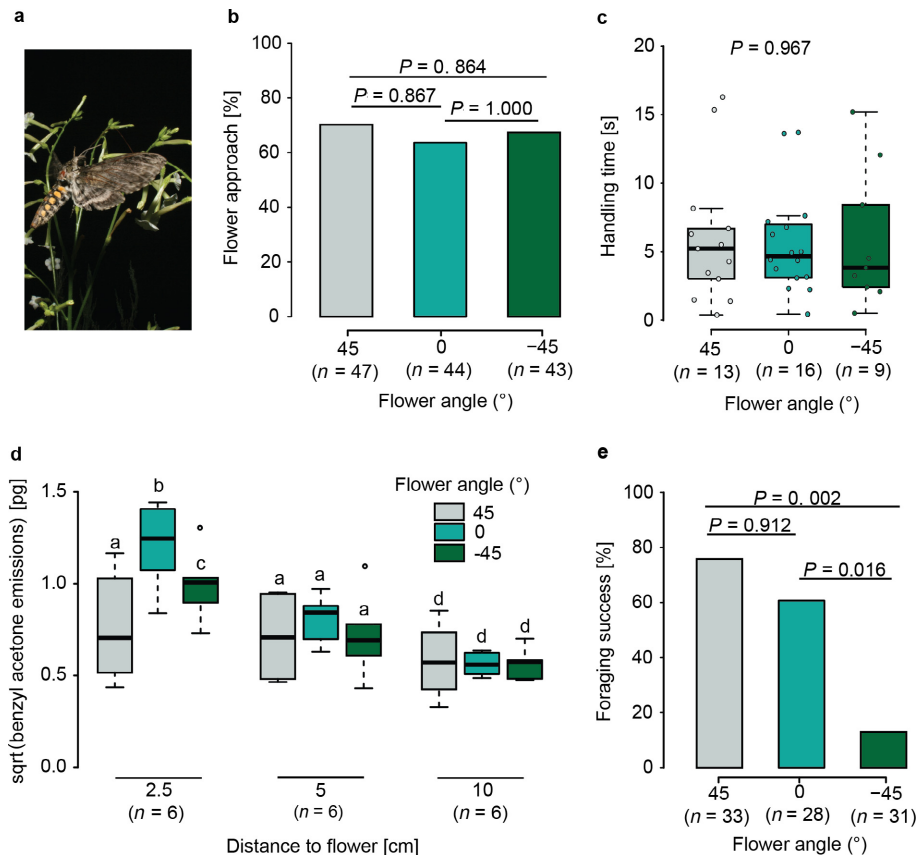


FIG. 1. Hawkmoths approached flowers at all orientations, but had higher success rates at upward and horizontally facing flowers. (a) *Manduca sexta* foraging at *Nicotiana attenuata*. (b) Moths visited the same number of flowers at an angle of +45°, 0° and -45°. (c) Handling times did not differ among flowers at different angles. (d) Absolute amount of benzyl acetone collected (pg) at the different flower orientations and at different distances from the corolla. Sqrt, square root. The amount of floral volatiles was strongly dependent on the distances from the corolla, but not on the flower orientation ($n = 6$ for each data point). (e) Foraging success, i.e., percentage of flowers that did not contain any nectar after a visit of a moth. Moths were significantly more successful in extracting the nectar from corolla tubes at +45° and 0° than at -45°. P values from a Holm-corrected Fischer's exact test are shown in panels b and e. Different lowercase letters show significant differences ($P \leq 0.05$) from a mixed linear model with distance and orientation as fixed factors with individual plants as random factor as shown in panel d. P value from a Kruskal-Wallis test followed by Holm-corrected Wilcoxon-rank sum test is shown in panel c. The box plots indicate median (mid lines), inter quartile range (boxes) and 1.5 times the inter quartile range (whiskers) as well as outliers (points).

MATERIALS AND METHODS

Manduca sexta moth

Moths were reared at the Max Planck Institute for Chemical Ecology (MPICE), Jena, Germany as described previously (Koenig et al. 2015); in short, eggs were obtained from female moths ovipositing on *N. attenuata* plants. After hatching, the caterpillars were transferred to a growth chamber with 27°C, 70% relative humidity (rH) and a dark:light cycle of 16:8 hours and fed an artificial diet (Koenig et al. 2015). For pupation, caterpillars at the last instar were placed individually into wooden blocks and kept under the same climatic conditions until 1 week before enclosure. Pupae were sexed and male and female animals were transferred to separate flight cages with a light regime of 15.5 h

daylight, 7.5 h moonlight, and two 0.5-h transition phases. Climatic conditions were set to 25°C and 70% rH during the daylight phase and to 20°C and 60% rH during the dark phase. For all experiments, we used male moths 72–76 h after enclosure.

N. attenuata plants

All plants were cultivated at the MPICE, Jena, Germany and originated from wild type *N. attenuata* plants, which were collected in a native population at the DI Ranch (Santa Clara, Utah, USA) and had subsequently been inbred for 31 generations. Seeds were sterilized and germinated on Petri dishes with Gamborg's B5 media as described in Krügel et al. (2002). Petri dishes with 30 seeds were maintained under LD (16 h light and 8 h dark) conditions in a growth chamber (Percival, Perry,

Iowa, USA) for 10 d, and seedlings were transferred to small pots (TEKU JP 3050 104 pots; Pöppelmann, Lohne, Germany) with Klasmann plug soil (Klasmann-Deilmann, Geeste, Germany) in a glasshouse. After 10 d, plants were transferred to 1-L pots and moved to a York Chamber (Johnson Controls, Cork, Ireland) with the same light regime and climatic conditions as the moth flight cages. In all cases, flowers were used 1–3 h after opening. Flowers were tethered at 45°, 0° or –45° angles in comparison to the horizontal plane, using a fine steel wire, coiled around the flower pedicel on one side and around the stem on the other side, in such a way that the angle was maintained but the flower remained sufficiently flexible to be moved by the moth. Angles were chosen based in the natural flower movement range of *N. attenuata*, which follows a 45° to –100° arch (Yon et al. 2016); we choose to use –45° to make a direct comparison to the upward-facing flowers.

Wind tunnel assays

In order to observe the foraging behavior of *M. sexta* moths on *N. attenuata* flowers at different orientations in detail, we recorded the visitation rate, handling time, and the foraging success of the moths at flowers with different orientations in a wind tunnel. The wind tunnel (250 × 90 × 90 cm) was run at a laminar flow of charcoal-filtered air at a wind speed of 0.37 m/s, 0.5 lux light, 25°C, and 70% rH, which is in the range of what is commonly experienced by foraging hawkmoths (Riffell et al. 2008). Moths and plants were placed into a separated pre-incubation chamber, set to the same conditions as the wind tunnel, at least 1 h before the experiment. A single plant with a single flower in a predetermined fixed orientation was placed into the tunnel directly before the experiment in such a way that the flower would be positioned 25 cm from the front end, 45 cm from each side, and 70 cm from the ground of the wind tunnel. The moths were kept individually in small mesh cages (height 15 cm, diameter 13 cm) and were introduced onto a platform at the rear end of the wind tunnel. Consistent with previous studies, all animals were then given 5 min to initiate wing fanning to confirm their active stage and to allow a warming-up of their flight muscles (Heinrich and Casey 1973, Haverkamp et al. 2016a, b, 2018). Moths that did not start fanning during this time were excluded from the experiment and not counted as replicates, resulting in a total of 43–47 moths that were analyzed per flower orientation. After take-off, the moths were allowed to fly freely in the tunnel for 4 min during which we observed the foraging behavior via a video camera (Logitech C615: Logitech, Lausanne, Switzerland, infrared filter removed) situated at the beginning of the wind tunnel directly behind the flower recording at 30 Hz and at a resolution of 800 × 600 pixels. Moths were scored as having approached a flower as soon as they contacted the flower with their proboscis or front legs and were scored as successful when they removed the floral nectar, which was

always measured after a moth had contacted the flower (see section Nectar measurement of flowers). We analyzed the flower handling time of those moths that had contacted the flower whenever the video sequence and quality was acceptable (9–16 moths per orientation). The time was counted from the first contact until the moth did not have contact anymore with the flower for more than 1 s. In order to exclude learning effects, we only considered the first flower handling of each moth for the statistical analyses. Moreover, we exchanged the plant after each trial where a moth had contacted the flower, to exclude any influence of the visited flower on the foraging behavior of the following moth.

Simultaneously, in order to test whether a change in flower orientation also affected the approach behavior of the moths we analyzed the last 2 s of the moth's flight before reaching the flower as described in a previous study (Haverkamp et al. 2016a). To do so, we recorded the flights of the moths via four video cameras and reconstructed the position of the animals at a rate of 10 Hz using a background subtraction method implemented in C (for review, see Sobral and Vacavant 2014). Only flights that were continuously tracked for the full 2 s were used for the analyses, leading to a slightly reduced sample size. Further analyzes and visualizations of three-dimensional flight tracks were performed using Matlab version 7.12 (Mathworks, Natick, MA, USA). All statistical analyzes were done using R version 3.4.0 (R Core Team 2017; packages stats [R Core Team 2017], lsmmeans [Lenth 2016], multcomp [Hothorn et al. 2008], and agricolae [de Mendiburu 2016]).

Pollen uptake and delivery

Flower orientation potentially not only affects the foraging success of the pollinator, but also the pollen transferred from and to a certain flower. To analyze whether flower orientation indeed changes pollen transfer by the moth, we first tested the amount of pollen that a moth would pick up from a flower at a certain orientation and second how much pollen was delivered during a given time period to a flower fixed at a certain angle.

For testing the amount of pollen picked up by the moth, the moth was captured directly after its 4-min flight in the wind tunnel and the proboscis was washed three times with 1 mL of a 1% Tween solution. Then 10 µL of a 0.5% safranin solution (Sigma Aldrich, St. Louis, MO, United States) were added to this proboscis wash to stain the sporopollenin. The samples were then vortexed and centrifuged for 2 min at 10,621 g and the supernatant was discarded. We then added 100 µL distilled water, vortexed again and pipetted 10 µL of each sample into a four-field Neubauer counting chamber (Superior Marienfeld, Lauda-Königshofen, Germany) to determine the number of pollen grains. Each sample was counted two times independently and the mean value was used for the total pollen estimation following the Neubauer chamber formula, in which the total number of pollen per proboscis equals the

pollen grains counted, multiplied by 10,000, divided by the number of counted fields and finally multiplied by a 0.1 dilution factor. We focused the pollen estimation on the moth's proboscis as this is the only body part of the moth that gets into contact with the flower during pollination as the corolla tube of *N. attenuata* (2.7 ± 0.4 cm [mean \pm SD]) is much shorter than the proboscis (7.5 ± 1.4 cm) and the pollen therefore mainly gets attached to the proboscis' tip.

To determine the influence of flower orientation on the pollen delivery to the plant, we analyzed the number of pollen grains that were delivered by a moth to the stigma of antherectomized plants. For this, we removed the anthers of still-closed flowers to avoid self-pollination by carefully cutting the corolla tube at the side and removing the anther heads with fine forceps (Kessler et al. 2008). Plants were then treated as described above and finally introduced individually into the wind tunnel. Before the start of the experiment, the proboscis of a moth was dusted with pollen collected the previous day, using a fine brush. On average, this method loaded 548.75 ($n = 41$, SE = 88.85) pollen grains to a single proboscis, which has previously been shown to lead to seed numbers comparable to those found under natural conditions (Kessler et al. 2015, Haverkamp et al. 2016b). The moth was then allowed to forage freely on the single antherectomized flower in the wind tunnel for 4 min or until the first successful foraging trial. After this, the stigmas were fixed and stored in 0.2-mL Eppendorf tubes using a FFA solution (one part formalin, eight parts 80% alcohol, one part acetic acid, by volume). Subsequently, stigmas were centrifuged to remove FFA solution, rinsed with distilled water and incubated in sodium hydroxide (8 mol/L) for maximum 4 h to clear tissues. The samples were centrifuged to remove previous solution and rinsed again with distilled water. Stigmas were placed in a staining solution with 0.1% aniline blue dye (fluorescence) and 0.1% safranin (bright field). Samples were removed from the staining solution, rinsed with distilled water and squeezed with the stigmas facing upward in a microscopy glass slide. Pollen grains in the stigma were then counted under a fluorescence microscope. Pollen uptake and delivery experiments were performed using two different sets of plants and moths to avoid cross transfer of pollen to flowers and moth learning effects.

Flower scent measurements

Flower volatiles from single flowers were trapped between 2 and 4 h after sunset in order to evaluate the scent distribution at each orientation, within a distance from the flower that is normally experienced by the antenna when the moth probes with its proboscis (~10 cm; Haverkamp et al. 2016a). We used 5 mm long polydimethylsiloxane (PDMS) tubes (inner diameter 1.5 mm, outer diameter 2.3 mm), during a 30-min collection time at 2.5, 5, and 10 cm distance from the corolla limb under the same wind tunnel conditions as

described in the wind tunnel assays section. At each experimental day, a single flower was used to trap the volatiles at each orientation, by manipulating the flower orientation in a random order. The samples were processed using a TDU-GC-MS as described elsewhere (Kallenbach et al. 2014). We added 11.8 pg bromohexane as an internal standard for quantification of the major floral volatile, benzyl acetone. The results were analyzed using a mixed-effects model, with distance from the flower and flower orientation as fixed factors and plant identity as random factor. The data were square-root transformed to ensure a normal distribution. All statistical analyses were carried out using R version 3.4.0 (R Core Team 2017).

Light exposure and pollen viability

Besides enhancing pollination, changes in flower orientation might also serve to protect the reproductive tissue from adverse environmental conditions. Here, we test how flower orientation might help to protect the flower pollen from heat radiation.

Plants were placed under a Sun-T Agro 400 W Na lamp (Philips, Amsterdam, The Netherlands). From each plant, three fully opened flowers at the first day after anthesis were chosen and oriented in the three different angles in the same way as described for the wind tunnel experiments. The limbs of the three flowers were kept ~15 cm away from the lamp. At this distance, flower temperature was comparable to those that had previously been measured at flower height of *Nicotiana* spp. in their natural habitat (mean = 36.9 °C, SD = 2.7, $n = 5$). The light spectrum emitted by the lamp, however, was mostly limited to a range between 540 and 840 nm (Appendix S1: Fig. S1), lacking most of the shorter wave length emitted by natural sunlight. Plants were treated under the lamp for 2 h. Anthers from the three treated flowers were collected and placed into Eppendorf tubes. Due to the static electricity, pollen grains attached to the wall of the tube, and the remaining parts of anthers could thus be removed by inverting the tubes. We added 200 μ L of freshly prepared liquid pollen tube growth medium (15 mmol/L MES pH 5.9, 1.0 mmol/L CaCl₂, 1.0 mmol/L KCl, 0.8 mmol/L MgSO₄, 1.6 mmol/L H₃BO₃, 0.005 mmol/L CuSO₄, 5% sucrose, 10% PEG 6000, 0.03% casein hydrolysate) into the tubes, which were then incubated in a growth chamber (Percival, Perry, Iowa, USA) at 26°C, dark for 3 h. After culturing, photos of the pollen tubes were taken under a Leica LMD6000 microscope (Leica Microsystems, Wetzlar, Germany) with a 6.3 \times objective in a transmitted light bright field set-up. For each culture, 5–10 fields were randomly chosen. Pollen tubes shorter than 35 μ m (the average diameter of pollen grain) were considered as non-germinated pollen grains.

Temperature at the anthers was measured by carefully cutting a flower at the side and inserting a k-type (chromel-alumel) thermocouple wire connected to a thermosensor

(MAX31856; Tinkerforge, Stukenbrock, Germany), in such a way that the uninsulated, temperature-sensitive part was placed at the position of the anthers (Fig. 2a). Temperature values were stored on a Raspberry Pi 3 computer (Raspberry Pi, Cambridge, UK).

Nectar measurement of flowers

In order to better quantify the foraging success at a *N. attenuata* flower of a certain orientation, we measured the remaining nectar in each flower, whenever a moth had contacted this flower during the wind tunnel assay. For this, we carefully removed the flower base and collected the nectar with a pre-weighed capillary. The nectar amount was determined by reweighing the capillary and subtracting the two measurements.

RESULTS

Hawkmoth behavior and floral volatile distribution

To investigate how flower orientation affects the moths' foraging and pollination behavior, we closely observed the interaction of *N. attenuata* flowers and their pollinators in a wind tunnel. We found similar proportions of hawkmoths approaching the flowers irrespective of the flower angle, indicating that flowers at all three orientations were equally detectable to *M. sexta* (+45° to 0°, $P = 0.867$, $n = 4,447$; +45° to -45°, $P = 0.864$, $n = 43-47$, $P = 0.867$; 0° to -45°, $P = 1$, $n = 4,344$; Fig. 1b). However, the flower orientation influenced the approach direction of the moth to some extent with the moths generally flying at a higher altitude, slightly above the plant, when the corolla was oriented upward or horizontally (F_{2,20} = 3.575, $P = 0.005$,

$n = 7-8$; Appendix S2: Fig. S1). Upon arrival, moths invested similar amounts of time into handling flowers at the different orientations (F_{2,35} = 0.031, $P = 0.97$, $n = 9-16$; Fig. 1c).

Since the handling time invested by *M. sexta* in a flower has been shown to strongly depend on the emissions of floral volatiles (Haverkamp et al. 2016b), we also investigated the distribution of floral volatiles at different flower orientations and at different distances from the flower (Fig. 1d). Here, we found a significant interaction between the flower angle and the volatile amount at different distances from the flower (L ratio [Likelihood ratio] = 12.176, $P = 0.016$, $n = 6$) in such a way that, for horizontally and downward-orientated flowers, the amount of odor decreased with increasing distance from the flower, while for upward-orientated flowers, no difference in the amount of odor at 2.5 cm distance and at 5 cm distance from the flower was found. However, the flower orientation on its own had no significant influence on the amount of flower volatiles detected (L ratio = 4.099, $P = 0.129$, $n = 6$), while instead the amount of odor collected was strongly altered by the distance from the flower (L ratio = 32.975, $P < 0.001$, $n = 6$).

Even though, in our study, *M. sexta* allocated similar amounts of time into flowers at all three orientations, they were less successful in gaining nectar from downward-facing flowers than from upward or horizontally oriented flowers (Fisher's exact test, $P = 0.002$ and $P = 0.016$ respectively, $n = 28-33$; Fig. 1e).

Pollination service and foraging success

The measurement of pollen grain uptake revealed that the moths had not picked up any pollen when they

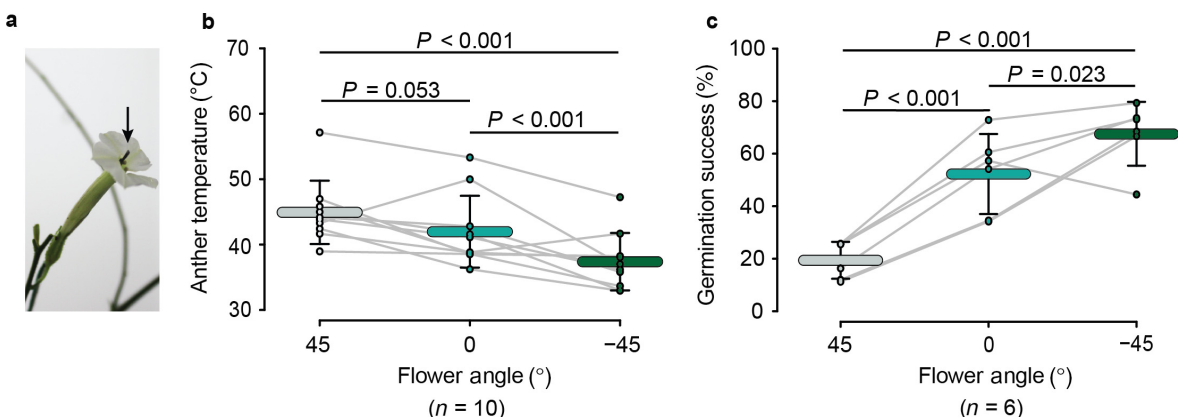


FIG. 2. Anther temperature and pollen germination success. (a) *N. attenuata* flower with wire thermometer (black arrow). (b) Temperature measured in flowers at three different angles (45°, 0°, -45°). Upward and horizontally orientated flowers had a significantly higher temperature at the anther level than downward-orientated flowers. Lines show mean values with error bars indicating the standard deviation, points show individual measurements in b and c. (c) Percentage of germinated pollen, collected from flowers held at three different angles (45°, 0°, -45°) at equal distance to a light and heat source. Pollen grains from flowers orientated at a positive angle (45°) had a significantly reduced germination rate in comparison to those from flowers orientated horizontally (0°) or downward (-45°). Light gray lines connect data points obtained from the same plant. P values from linear mixed-effect model followed by Tukey-corrected multiple comparisons of means are shown in panels b and c.

handled flowers with downward orientations whereas moths foraging on horizontal or upward-oriented flowers received between 1,625 and 2,125 pollen grains per trial ($\chi^2 = 12.87$, $P < 0.02$, $n = 7-9$; Fig. 3c). Additionally, the counting of pollen grains on the stigma after a single visit (Fig. 3a), showed that the moths delivered 10 and 14 times more pollen grains to the stigma of upward and horizontally oriented flowers than to downward-facing ones ($\chi^2 = 6.928$, $P = 0.031$, $n = 17-21$; Fig. 3b). In parallel, the analysis of nectar amounts showed that in case of an upward and horizontal flower orientation, moths were able to empty the flower in nearly all cases, whereas significantly more flowers with remaining nectar were found after the moths had to handle downward-oriented flowers ($\chi^2 = 24.717$, $P < 0.001$, $n = 25-27$; Fig. 3d), probably due to their low success rate in inserting their proboscis into downward orientated flowers (Fig. 1e). In those cases in which the moths did not visit the flower, we found on average 2.32 mg (SD = 1.24, $n = 36$) of nectar in the corolla tube with no difference between flowers at different orientations (ANOVA, $F_{2,33} = 0.116$, $P = 0.891$, $n = 5-22$).

Consequences of flower orientation for anther temperature and pollen viability

Here, we measured the temperature in close proximity to the flower anthers, finding that upward orientation increases temperature by 7.5°C in comparison to the downward orientation from 37.4°C to 44.9°C, while a horizontal orientation caused an increase of 4.6°C to 42°C (linear mixed-effect model, $F_{1,2} = 17.735$, $P < 0.001$). The germination rate of individual pollen grains was examined to assess the effect of a higher heat exposure on upward-oriented flowers. We found that heat levels, which commonly occur in the desert habitat of *N. attenuata* (midday air temperatures during the flowering season [May 2018] of *N. attenuata* at DC Range in Utah, USA reached 45.3°C), significantly reduced the germination rate of pollen grains from an upward-oriented flower in comparison to the horizontal and downward orientations. Flowers at a horizontal orientation also had a significantly lower pollen germination rate than flowers at a downward orientation (linear mixed-effect model, $F_{1,2} = 35.7119$, $P = 0.023$),

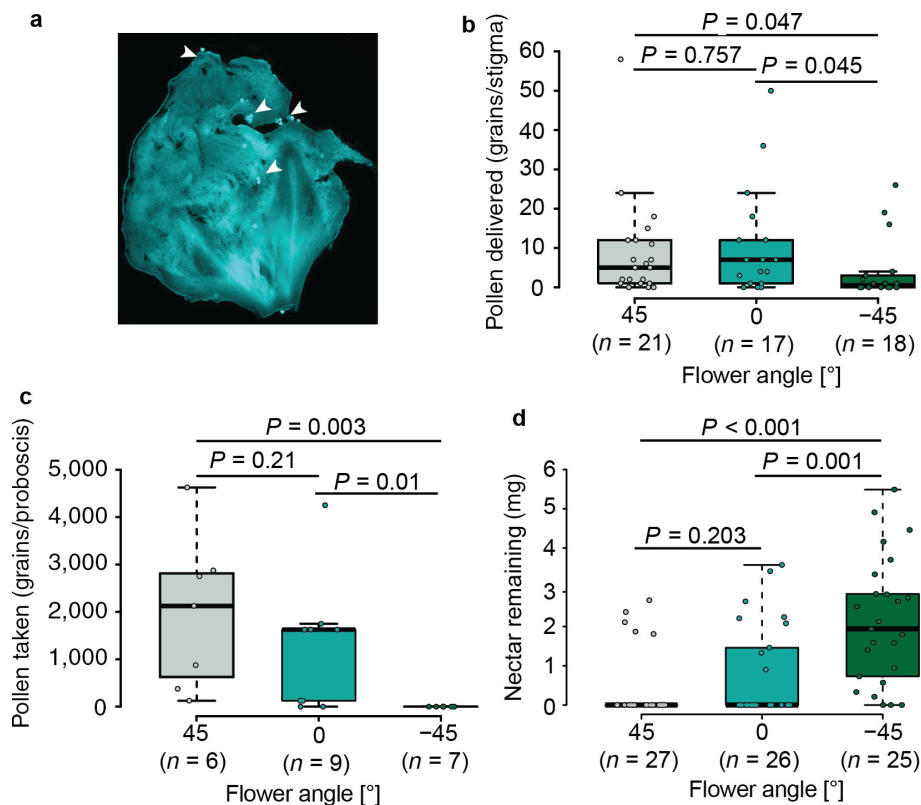


Fig. 3. Flower orientation influenced both pollen uptake and pollen delivery. (a) Pollen grains (arrows) on the stigma of *N. attenuata* after pollination by *M. sexta*. (b) Moths with similar pollen loads on their proboscis delivered significantly more pollen to stigmas of emasculated flowers fixed at 45° or 0° angles than to flowers at a -45° angle. (c) More pollen was taken up by moths foraging on flowers at +45° and 0° angles. (d) Less nectar remained in flowers at an angle of 45° than at any of the lower angle positions after the moths had contacted and attempted to feed, indicating a higher foraging success of the moths at this angle. P values from a Kruskal-Wallis test followed by Holm-corrected Wilcoxon-rank sum test are shown in panels b, c, and d.

indicating a strong influence of flower orientation on pollen viability.

DISCUSSION

The coevolution of plants and their pollinators is often argued to be a major driving force of plant and insect diversity (Johnson and Steiner 2000). The present study identifies flower orientation as an important aspect in the interaction and co-adaptation between *N. attenuata* and its long-tongued hawkmoth pollinators such as *M. sexta*. It also highlights the importance of a reliable match between flower morphology and flower signals for this plant–pollinator relationship. Moreover, our analysis indicates that the plant not only actively moves its flower to a position optimal for pollination, but also reverses this movement and thereby avoids damage to the flower through abiotic stress.

Upward flower orientation clearly aided *M. sexta* and we can expect similar benefits for other long-tongued hawkmoth pollinators with a similar behavior as well as morphology such as *M. quinquemaculata* or *M. rustica*, which also occur frequently in the habitat of *N. attenuata* (Alarcón et al. 2008). However, foraging of other *N. attenuata* pollinators such as the short-tongued hawkmoth *Hyles lineata* or different hummingbird species might be less impaired by different flower orientations, due to their higher maneuverability (Maglianesi et al. 2015). In the presence of these pollinators, plants could therefore potentially save the costs of flower movement and receive similar pollination service (for energy estimations see Appendix S3). Yet, especially hummingbirds have been argued to be less effective pollinators of *N. attenuata* as they mainly forage in the close vicinity of their nest and often visit multiple flowers on the same plant, presumably leading to a lower inter-individual pollen dispersal rate and higher geitonogamy, particularly as *N. attenuata* grows in clumps of simultaneously opening flowers (Canela and Sazima 2003, Kessler et al. 2010).

In addition to the amount of pollen transferred to the proboscis (Fig. 3c), the present results also highlight the importance of pollination efficiency. Flower orientation played an important role in how effectively pollen had been picked up by hawkmoth pollinators and delivered to the stigma of *N. attenuata* flowers. The near lack of pollen delivery to flowers at a downward orientation indicates that independently of the capacity to reach the flower it does also matter from which direction the pollinator contacts the flower upon inserting the proboscis, as otherwise the pollen might not be deposited on the stigma (Fig. 3b). In contrast to pollen delivery, one could hypothesize that the removal of pollen would be easier as most anthers shed their pollen upon contact and/or vibration; however, our data demonstrate that simply reaching the flower is not sufficient for pollen removal (Fig. 3c).

The genus *Nicotiana* includes many species with floral traits matching those of a hawkmoth pollination

syndrome, indicating that the adaptation to hawkmoth pollinators might also be under phylogenetic constraints that cannot be easily altered (Raguso 2006, Raguso et al. 2006, Tiedge et al. 2017). This hypothesis is further supported by the finding that independent of their pollinators many *Nicotiana* species emit more benzenoid volatiles at night, which are highly attractive to hawkmoths (Raguso et al. 2003). Notably, flower orientation and floral volatile emissions in *N. attenuata* are under the influence of the same circadian genes, which ensure the synchronization of flower angle and odor production with the moths' night activity, which can be considered as one part of several adaptations to hawkmoth pollination (Yon et al. 2016).

More specialist pollinators such as *M. sexta*, often rely on certain floral advertisements (i.e., a specific flower scent) to make their foraging decisions (Schiestl and Johnson 2013). In addition, most pollinators also memorize flower features such as color or scent in a multimodal and reward dependent manner in order to make subsequent foraging choices (Waser et al. 1996, Wright et al. 2009, Riffell and Alarcón 2013, Riffell et al. 2013). The foraging behavior of the hawkmoth *M. sexta* has been shown to be strongly influenced by floral volatiles (Haverkamp et al. 2016a). This olfaction dependency should make the moth potentially vulnerable to changes in the air vortices that occur around the flower, i.e., through changes in floral orientation, which potentially could affect the scent plume structure (Ortega-Jimenez et al. 2013). However, we found equal number of moths approaching flowers at different orientations (Fig. 1b), suggesting that the floral scent plume was not affected considerably. Additionally, flower orientation might have also changed the visibility of the flower to the moths. However, if this was the case, it did not influence the overall attractiveness of the plant, even though we did find a difference in the approach altitude of the moths toward flowers at different orientations, likely caused by a difference in flower visibility (Appendix S2: Fig. S1).

Interestingly, the hawkmoth *H. lineata* was able to select upward facing flowers of *Aquilegia pubescens* at a distance when these were directly compared with downward-facing flowers in a paired-choice assay (Fulton and Hodges 1999). This suggests that the day and night active *H. lineata* might rely more on visual cues (Kessler et al. 2015), which would allow a better discrimination between flower orientations and make the moth less dependent on a single floral signal. Similarly, different species of African hawkmoths preferred to first probe upward-oriented flowers of the fly-pollinated *Zaluzian-skya microsiphon*, which does not emit any floral scent attractive to hawkmoths, forcing the moth again to rely on visual information (Campbell et al. 2016).

Additionally, several studies suggest that *M. sexta* has not only adapted its sensory system toward the flowers it pollinates (Riffell et al. 2013, Haverkamp et al. 2016a), but also its movement patterns (Sponberg et al. 2015). *M. sexta* usually maintains a body angle of $34.3^\circ \pm 3.3^\circ$

(mean \pm SD) with the head higher than the thorax while hovering in front of a flower (Hedrick and Daniel 2006). Thus, moths might be reluctant or not able to take a steeper body angle as this will make hovering flight too unstable for efficient foraging (Willmott and Ellington 1997). Although some Lepidoptera appear to be able to lift their proboscis tip above their body axis (Krenn 2010), we did not observe this for *M. sexta* in our video recordings, limiting the maximum downward orientation of a flower on which moths can forage to its body angle. For moths with shorter proboscises, this limitation could be bypassed by an increased body or proboscis maneuverability (Farina et al. 1994), or by being capable of landing on flowers that can support their weight (Ushimaru and Nakata 2002). Other studies that investigated the influence of more rapid flower movement induced by wind found that *M. sexta* was better able to follow a moving flower along a horizontal arc than a flower that moved along a similar arc but back and forth (Sprayberry and Suver 2011). Interestingly, measurements in several hawkmoth pollinated flowers found that these flowers more often move along those directions and at a frequency, which can be well tracked by the moth. Hence, the flight abilities of *M. sexta* do indeed appear to correlate with those movements that are most likely to occur in flowers pollinated by long-tongued hawkmoths (Sprayberry and Suver 2011, Sponberg et al. 2015).

In addition to nectar production, flower movement is another investment the plant makes to ensure pollination. This flower movement is likely achieved through permanent asymmetric cell growth, on either the abaxial and adaxial flank of the pedicel, and/or through an active temporary change in turgor pressure, making both the upward and the downward movement of the flower a costly process (Uehlein and Kaldenhoff 2008). This angular movement in the pedicel is unusual since the changes caused in the abaxial and adaxial flanks are strong enough to change flower orientation from upward to downward, which are different from the subtle circular or one-time reorientation of many other flower pedicels (Webb 1984, van Doorn et al. 2013, Vandenbrink et al. 2014).

The nectar of *N. attenuata* contains on average 8.59 J (Haverkamp et al. 2016a). We estimate, based on the flower mass and the angular distance moved by the flower, that the daily change in flower orientation consumes about 1.62 J of energy (see Appendix S3: Fig. S1). This would mean that the flower movement might take about 20% of the energy a plant normally invests into nectar calories, taken aside other costs for producing and transporting the nectar (Heil 2011). Considering that this daily flower movement increases the successful interactions between the moth and the flower by nearly 62%, moving the flower to an upward orientation appears to be an economically efficient investment for the plant, even if we assume that this change in orientation would only occur to aid pollination. Investing into an upward position of the flower appears especially effective as

experiments with nectar-less mutant plants have shown that floral nectar on its own increases pollination success by *M. sexta* only by about 55% (Kessler et al. 2015). However, as mentioned earlier, all these energetic investments will only pay off in the presence of *M. sexta* or similar pollinators while, in habitats without these flower visitors, plants should not perform this change in orientation. Interestingly, some populations of *N. attenuata* have recently been shown to be deficient in the production of benzyl acetone, the major compound used by the flower to attract *M. sexta*. It has been argued that in these populations, either the density of *M. sexta* is too low to merit the production of this compound or that the costs of attracting female moths, which might also oviposit on these plants, outweighs the pollination benefits (Haverkamp et al. 2018). Given these findings, it would indeed be highly interesting to test whether *N. attenuata* populations that do not produce any benzyl acetone also do not move their flowers to an upward orientation and whether they employ alternative ways of pollination.

The present results also show that heat radiation reduces pollen viability by almost 50% (Fig. 2c), again increasing the fitness return of flower movement. Thus, the downward orientation of flowers during the day has likely evolved to protect the plant reproductive tissue from adverse environmental conditions. However, in this study, the artificial light source used to mimic the abiotic conditions of the desert environment of *N. attenuata*, only provided realistic temperature conditions, but it did not produce UV-B radiation. As the pollen of upward orientated flowers will likely be more exposed to UV-B radiation this might have even further enlarged the differences we have observed here (Demchik and Day 1996). In addition to abiotic stress, the downward orientation of the flower during the day, might also help the plant to hide from day active nectar robbing bees (i.e., carpenter bees), which might be visually attracted to the upward-oriented corolla and are known to damage about 10% of all *N. attenuata* flowers in the field (Kessler et al. 2008). Hence the downward orientation in the flower might offer a double function of not just protecting pollen but also avoiding nectar robbers, similar to what has been proposed for the red color of hummingbird-pollinated flowers, which are also less visible to Hymenopteran nectar robbers (Forrest and Thomson 2009).

Taken together, the present results highlight the importance of synchronizing the emission of floral volatiles with flower orientation for a successful interaction between *N. attenuata* and its hawkmoth pollinator *M. sexta*. Our study further suggests that flower traits might often be shaped by a balance between pollinator attraction and protection from the abiotic or biotic environment. Identifying such potentially contrasting selection pressures might lead to new insights into the evolution of flower characteristics and could thereby enhance our understanding of the vital interaction between plants and their pollinators.

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SUPPORTING INFORMATION

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DATA AVAILABILITY

Data are available from the Dryad Digital Repository: <https://doi.org/10.5061/dryad.4769955>