

Innate Adjustment of Visitation Behavior to Rewarding and Reward-Minimized *Petunia axillaris* (Solanacea) Plants by Hawkmoth *Manduca sexta* (Sphingidae)

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Received: October 17, 2011 Initial acceptance: December 4, 2011 Final acceptance: April 26, 2012 (M. Herberstein)

doi: 10.1111/j.1439-0310.2012.02055.x

Abstract

Plant-pollinator interactions offer an excellent system to study the stability of mutualistic interactions. While nectar production requires resources and a reduction could in principle benefit plant fitness, only few angiosperms lack nectar, and thus cheat from a pollinator's perspective. Cheating behavior may be scarce because of pollinator foraging behaviors that select for nectariferous plants. Shorter inspection duration, interaction with fewer flowers, or even complete avoidance of plants with low/no nectar may reduce the fitness of cheating plants. The effectiveness of pollinator strategies may depend on how they are implemented. Innate strategies would invariably decrease the fitness of a cheating plant, while learned responses allow cheaters to exploit naïve pollinators. Here, we studied the foraging strategies of the hawkmoth Manduca sexta during interactions with nectariferous and reward-minimized Petunia axillaris. We found that neither naïve nor experienced hawkmoths discriminated a priori between rewarding and nectar-less plants. However, naïve hawkmoths displayed reduced probing time per flower and number of flowers visited on reward-minimized plants during the first trial, without showing further improved discrimination with experience. In conclusion, the foraging decision rules of hawkmoths that may reduce the fitness of rewardminimized plants appear to be innate, with little scope for additional learning.

Introduction

Mutualisms are interactions between species, which result in a net fitness increase of both partners. They are likely to play an important role in nearly every ecosystem (Bronstein 1994). The conditions for the evolution and persistence of mutualistic interactions are subject to great research efforts (Bshary & Bronstein 2004; Sachs et al. 2004; West et al. 2008) because exploitation by other species and by the socalled cheaters may destabilize mutualisms (Doebeli & Knowlton 1998; Hoeksema & Bruna 2000; Bronstein 2001a,b). Several strategies called partner control mechanisms can reinforce investment and therefore maintain mutualisms, such as partner choice (a preferential selection of a suitable partner to interact with; Bull & Rice 1991; Noë 1991) and sanctions (partners cut back on provisioning the traded good; Herre et al. 1999). The effectiveness of these mechanisms may depend on how they are implemented. Innate strategies such as recognition and discrimination of petal coloration by insects work immediately (e.g., Lunau et al. 1996), while learning (experience-based modification of behavior) may delay the expression of appropriate behavior and hence offers opportunity for cheating naïve individuals (Gaskett 2011).

Plant–pollinator mutualisms are suitable systems to explore the potential importance of learning vs. innate strategies in the maintenance of mutualisms. In these mutualistic interactions, pollinators forage for nectar and pollen on flowering plants, thereby distributing the plant's pollen as a result of self-serving behavior. The plants produce nectar as a reward. The costs of nectar production appear to be quite variable. Some studies report considerable negative effects of nectar production on a plant's reproductive potential (Southwick 1984; Pyke 1991; Brandenburg et al. 2009) while other studies fail to find any effects (Leiss et al. 2004). The costs of nectar production will probably strongly depend on a plant's exposure to sunlight (Cawoy et al. 2008). If nectar production was costly, any means to reduce nectar production without compromising pollination should be under positive selection. If nectar production is rather cost free, one nevertheless has to ask how pollinator foraging rules select for the production of stable amounts of nectar, on a level that maximizes plant fitness while maintaining the mutualism with the pollinators. For the purpose of this manuscript, we take the perspective of pollinators and hence refer to plants that provide little/no nectar as cheaters, independently of whether these plants experience energetic benefits from that. Only approximately 4% of angiosperms have evolved into being cheaters from a pollinator's perspective (Renner 2006), most notably deceptive orchids (Schiestl 2005; Gaskett 2011). In the case of orchids, interactions with naïve individuals are suggested as a major hypothesis to explain the persistence of cheating (Smithson & Gigord 2003).

Three behaviors of pollinators have been identified that could potentially reduce the fitness of cheating plants: avoidance of non-rewarding species (Gigord et al. 2002), reduction in probing time (Cresswell 1999), and reduction in number of flowers visited on cheaters (Ohara & Higashi 1994; Smithson & Gigord 2001, 2003). Two points are of importance. First, low nectar volumes (reward-minimized flowers) are regularly encountered because of the activity of other pollinators. From a pollinator's perspective, it does not matter whether low foraging success is attributed to the plant cheating or the previous visits by other pollinators. Thus, behaviors that help pollinators to generally reduce the time spent in low-quality food patches should evolve even in the absence of cheaters. Second, the effects of pollinator strategies to maximize foraging success will differ between cheating and nectar-providing plants. The latter obtain the benefits of nectar provisioning (increase in foreign pollen deposition and/or an increase in pollen uptake) from the first pollinator and potentially other ones after replenishment of nectar while cheating plants will invariably suffer from the pollinators' decision rules. Note that despite generally lower fruit sets, some deceptive orchids might actually benefit from a lack of nectar by increasing outcrossing rates and gene flow (Cozzolino & Widmer 2005).

To date, most research efforts on cheating in plantpollinator systems predominantly address either the issue of cheating species or cheating in sexually dimorphic species (Ashman et al. 2005: Pohl et al. 2008; Ashman 2009). In these cases, important flower characteristics differ between cheaters and cooperators. Therefore, experiments on the role of learning in pollinators have tested for the ability to distinguish between cues that typically belong to different species or morphs such as colors, flower shapes, or scents. A great number of excellent studies demonstrate how pollinators construct an internal representation of the outside world via their sensory filters and use this information to make future decisions about revisiting or avoiding flowers with particular cues (Weiss 1991; Hammer & Menzel 1995; Chittka et al. 1999; Menzel 1999; Chittka & Thomson 2001; Chittka & Raine 2006; Goyret et al. 2008a,b; Ashman 2009; Rodrigues et al. 2009; Wright et al. 2009). However, these studies are not conclusive regarding potential pollinator responses to rewardless (cheating) mutants displaying identical phenotypes within a population of nectarproviding plants when changes in pollinator behavior may reduce the cheaters' fitness.

Here, we investigated how the tobacco hornworm moth Manduca sexta responds to reward-minimized (cheating) Petunia axillaris. Hawkmoths appear to be good at controlling cheating as they are seldom partners of cheating plant species (Renner 2006). Manduca sexta is innately attracted to Petunia axillaris as a food source, while P. axillaris does not serve as a host plant for oviposition. Over consecutive trials, we exposed hawkmoths simultaneously to one plant with nectar and one with manually removed nectar. Plants were exchanged between trials but locations of rewarding and reward-minimized plants were kept constant. We asked whether hawkmoths could avoid, reduce probing time, and visit fewer flowers on rewardminimized plants. For any shown discriminative ability, we predicted that if it were innate, it would be evident from the very first foraging event. In contrast, if learning plays a role, the discriminative ability of *M. sexta* will improve over successive learning trials.

Methods

All experiments were conducted in a greenhouse of the Institute of Plant Science, University of Bern, from August 2006 until October 2006. *Manduca sexta*, a nocturnal hawkmoth, is a natural pollinator of several solanaceous species including *Petunia axillaris*. Female pupae of *M. sexta* were obtained from NCSU Insectary (Raleigh, NC, USA) and kept in BugDorm-3[®] insect

tents at 24°C, with 60% air humidity and a 16/8 day/ night cycle. Pupae were controlled daily for eclosion of adults that were subsequently used for the experiments. Adults emerged 3–5 d before the experiments and were starved prior to use. Hawkmoths were completely naïve and were used unmated for experiments.

The plant species used for experiments was *Petunia axillaris axillaris* N (later referred to as *P. axillaris*), a self-compatible inbred line (kindly provided by Ronald Koes, Vrije Universiteit Amsterdam), derived from a wild accession of *P. axillaris axillaris*. This line has been maintained by inbreeding in the Institute of Plant Science, University of Bern. Flowers of *P. axillaris* display all characteristics of a hawkmoth-pollination syndrome (sensu Faegri & van der Pijl 1979): large white petals, sweet scent emitted at dusk, long floral tube filled with large amounts of nectar. Plants were grown in peat-based soil, in 15-cm-diameter plastic pots and kept under greenhouse conditions (supplementary light in winter months, minimum 14 h light).

In our hawkmoth experiments, we used plants that contained the full nectar reward (further labeled 'with nectar') and plants where nectar was manually removed so that the reward was minimized. To extract nectar from 'reward-minimized' treatment group, the floral tube was pierced at the bottom of the floral tube, and exuding nectar was removed with a tissue. To avoid replenishment, nectar was removed hourly. Such treatment yields clearly distinct nectar volumes between treatment groups (Brandenburg & Bshary 2011). To exclude that the tissue injury would elicit some kind of behavioral response in the pollinator, the control plants were also pierced in the floral tube, but above nectar levels. No scent difference could be detected between 'with nectar' and 'rewardminimized' treatment in isolated flowers 30 min after cutting (Brandenburg & Bshary 2011).

Set-up of Behavioral Experiments with Manduca sexta

The experiments were conducted in a flight arena (144 cm height, 248 \times 368 cm surface area), situated in the middle of a greenhouse used for growing Petunias, and therefore scent-saturated. Experiments started at around 1700 (winter) and 2030 (summer) and ended latest at 2300 and were conducted by a single investigator. Hawkmoths were kept in flight cages (BugDorm) before the onset of experiments. For pollinator observation, the flight arena was illuminated with a shaded 15-V incandescent light bulb. Light intensity was 0 µmol m²/s measured by a quantum light sensor model 3668I (Spectrum® Technologies,

Inc., Bridgend, UK). We could not determine an orientation toward the light source.

One plant of each treatment group ('nectar minimized' and 'with nectar') was placed 1.7 m from one another in the flight arena and presented simultaneously to the pollinator. Each hawkmoth was tested three times per night on three consecutive nights, each time exposed to a set of new plants. A total number of 21 hawkmoths was used for the general linear model. Previous studies conducted with another hawkmoth species, Macroglossum stellatarum (Kelber & Henique 1999), demonstrated that 1-10 trials were required to learn the link between a color and reward. The positioning of 'reward-minimized' and 'with nectar' plants was kept identical throughout the experiment. The flight arena had three different entrance sites, which were chosen in a counterbalanced way between nights to exclude that the hawkmoths could develop a side bias. One insect was released into the flight arena at a time, and the following behaviors were recorded: (1) First choice - noted as the plant ('reward-minimized'/'with nectar') that hawkmoths first probed on. (2) Number of flowers visited - total number of flowers on each plant that hawkmoths probed from. (3) The probing time per flower was recorded from the insertion of the proboscis until its retraction. For the last two behaviors, only the first probing event on each flower was noted. The probing duration was measured with a chronometer. All behaviors were recorded with a Dictaphone and analyzed the following day. We set a maximal time interval of 300 s for the pollinators to interact with the plants in each trial, after which plants were exchanged and the next trial began. Hawkmoths that failed to interact with either plant on the very first trial were not used further. The plant exchange process was realized within a few seconds, during which the hawkmoth remained in the flight arena. Hawkmoths were removed after the three trials and returned to their cages until the next night.

Data Analysis

Global analyses

We calculated GLM repeated measures to see (1) whether there are significant differences between 'number of flowers visited' and 'probing time' on 'with nectar' and 'reward-minimized' plants with all data combined and (2) whether the performance of subjects changed with the duration of the experiment. The latter analyses were used to test for a general improvement in performance like a reduction in handling time, as well as to test for improvements in

discrimination as an indicator of learning. To test whether subjects could a priori distinguish between 'with nectar' and 'reward-minimized' plants, we summed for each hawkmoth the first approach over all trials (one pool 'reward-minimized', one 'with nectar') and calculated a Wilcoxon signed rank test instead of a GLM because of the binomial nature of the raw data.

First trials

For the variables that would yield overall significant differences in hawkmoth behavior toward plants 'with nectar' compared with plants 'reward-minimized' in the global analyses, we calculated Wilcoxon signed rank tests to assess whether these differences were already manifested in the very first trial.

The statistical analyses were conducted using SPSS 17.0 (SPSS, Inc., Chicago, IL, USA). To analyze the differences in the number of flowers visited and the probing time across the different trials between rewarding and non-rewarding plants, a repeated measures ANOVA was used with nectar production as fixed factor and the number of flowers and probing time across the different trials as the repeated measure. PROC GLM with a repeated statement was used to perform the overall analysis, determine sphericity (Mauchly's test) and to generate univariate results.

Results

There was a significant difference between 'with nectar' and 'reward-minimized' plants in probing time and number of flowers visited in all trials (GLM repeated measures, $F_{\text{with/min}} = 28.28$, p < 0.001, Figs 1 and 2, Table 1). There was no significant difference in first-choice behavior of each hawkmoth over the course of the experiment (Wilcoxon signed rank test, N = 21, Z = -0.591, p = 0.555, Fig. 3).

Hawkmoths probed significantly shorter and visited significantly fewer flowers on 'reward-minimized' plants than on 'with nectar' plants already on the very first trial (Wilcoxon signed rank tests; probing time: Wilcoxon signed rank test, N = 18, Z = -2.201, p = 0.03; number of flowers visited: N = 18; three hawkmoths did not visit plants during the first trial, Z = -2.578, p = 0.01, Figs 1 and 2).

Over the course of trials, both probing time and number of flowers visited were significantly reduced in both between-subjects categories ('with nectar' and 'reward-minimized'; $F_{1,9} = 4.66$, p < 0.001). There was no interaction between the subjects ('with nectar'/'reward-minimized') over the course of the



Fig. 1: Mean probing time per flower (+/- s.e.) across 9 trials. The nine trials take place over the course of three nights (three trials per night). The black circles indicate the probing duration of hawkmoths on *P. axillaris* plants with regular nectar amounts, while the gray circles indicate the probing duration of moths on *P. axillaris* plants where nectar has been manually removed.



Fig. 2: Mean number of flowers per plant visited by pollinators (\pm s.e.) across 9 trials. The nine trials take place over the course of three nights (three trials per night). The black circles indicate the number of flowers visited by hawkmoths on *P. axillaris* plants with regular nectar amounts, while the gray circles indicate the number of flowers visited by moths on *P. axillaris* plants where nectar has been manually removed.

experiment, neither in the category probing time (F = 1.36, df = 4.5, p = 0.25, Fig. 1) nor in the category number of flowers visited (F = 1.32, df = 5.9, p = 0.25, Fig. 2).

Discussion

The aim of this study was to determine (1) any spontaneous foraging decision rules of *M. sexta* faced with *Petunia axillaris* plants offering normal amounts of nectar and reward-minimized (nectar removed) plants that may act as control mechanisms against cheating and (2) the scope for learning in response to exposure to reward-minimized plants. We investigated three features of pollinator foraging behavior that might select for nectar production in plants: the



Fig. 3: Percentage of hawkmoths that select the rewarding plant as first choice across the 3 trial nights, represented as boxplots with median, 1st and 3rd interquartile. The dashed line indicates the null hypothesis that hawkmoths select 50% rewarding and reward-minimized as their first choice, because of the assumption that flowers of both treatments display no phenotypic differences.

Table 1: Results of a repeated measures analysis of variance for the effect of nectar on the number of flowers probed and probing time

Effect	Value	F	Hypothesis df	Error df	Sig.	$ {Partial} \\ \eta^2 $
Between subjects						
Nectar (with/no)	0.592	28.282	2	39	0.000	0.592
Trials	0.749	4.656	16	25	0.000	0.749
Within subjec Trials × nectar	ts 0.530	1.759	16	25	0.100	0.530

ability to identify and avoid reward-minimized plants prior to probing, a decision rule to increase the likelihood of leaving when probing a reward-minimized flower, and shorter probing times on reward-minimized flowers (Smithson & Gigord 2003).

Our results demonstrate that naïve individuals reduce the probing time per flower and are more likely to end the interaction with a plant when encountering an empty flower. Thus, these decision rules appear to be innate. Both strategies help to improve foraging efficiency considering that previous floral visitors may have already depleted a food patch. Simultaneously, these foraging rules may act as partner control mechanisms, as nectar-providing plants will be searched for longer while still containing nectar whereas nectar-less plants will always experience short encounters. Thus, decision rules that cause pollinators to increase foraging efficiency may also select for plants that provide nectar.

In accordance with field results (Brandenburg & Bshary 2011), we found no evidence for prior avoidance of 'reward-minimized' plants. The inability to discriminate empty from otherwise identical rewarding flowers prior to probing seems to be a common pattern of foraging insects (Thakar et al. 2003). As nectar is mostly concealed within the plant and visually not accessible for pollinators, the content cannot be evaluated before the insertion of a feeding organ (Cnaani et al. 2005). Nectarless (deceptive) orchids exploit this by luring naïve pollinators to attractive floral displays or mimicked mating signals (Dafni 1984; Schiestl 2005; Gaskett 2011). The time frame where nectarless orchids can reproduce is limited to the state of naivety of pollen vectors and significantly decreases with increased experience of pollinators (Ferdy et al. 1998; Gigord et al. 2002; Internicola et al. 2007). Both visual (Weiss 1997) and olfactory cues such as CO2 (Guerenstein et al. 2004; Thom et al. 2004; Riffell et al. 2008) may be involved in learned avoidance of cheater species/phenotypes. However, learning to avoid cheaters is constrained if mimics closely resemble the phenotype of model nectariferous plants (Dver & Chittka 2004; Internicola et al. 2007). This would certainly be the case of a cheater plant representing a mutation in an otherwise mutualistic lineage, as simulated in our experiment. The scent profile remained similar after nectar extraction (Brandenburg & Bshary 2011) and selection would favor cheaters that specifically reduce nectar production without changing anything else. Thus, we consider it unlikely that prior avoidance of intraspecific cheaters will be an important partner control mechanism.

We did not find any evidence that hawkmoths improved their performance after repeated exposure. As the moths adjusted probing duration and number of flowers visited to nectar volumes from the very beginning, further improvement through learning may be of relatively minor importance for Manduca sexta. In contrast, one could have expected that subjects learn to avoid a location where nectar rewards are minimized but this was not the case. Studies in other contexts and on other species have shown that learning about food sources can be achieved in one to few trials (Dukas & Real 1993; Hammer & Menzel 1995; Kelber & Henique 1999; Daly et al. 2001; Healy & Hurly 2001; Balkenius et al. 2004; Balkenius & Balkenius 2010). We cannot exclude the possibility that hawkmoths performing in our experiments learned much slower than expected and demonstrated in other studies. However, slow learning would only have a small negative impact on cheating plants and hence already small benefits of nectar reduction would become more likely to be under positive selection. In experiments with free-flying hawkmoth *Macroglossum stellatarum*, few trials (1–10) were required to establish associative learning of spectral colors (Kelber & Henique 1999), and we used this value as a reference for our own experiments. Generally, hawkmoths seem to have a strong innate sensory bias for certain cues that may help to learn profitable plant species quickly but that can inhibit learning in the context of identifying cheaters within a plant population (Kelber 2002, 2010; Balkenius et al. 2008).

In field experiments with hawkmoths of unknown experience, we had observed that individuals in a high-density population of wild petunia responded to nearly empty flowers with switching to another plant while such decision rule could not be documented in a low-density population of wild petunia (Brandenburg & Bshary 2011). One possible interpretation of these results is that hawkmoth in the low-density petunia population learned to be less discriminative because the costs of switching between more spaced plants may outweigh the risk of probing more flowers that may also be empty.

More generally, learning is important in other aspects of the hawkmoths' life, such as host plant choice (Cunningham & West 2008), odor learning (Daly & Smith 2000; Daly et al. 2001), or flower handling (Goyret & Raguso 2006). In accordance with the previous studies, we found a decline in probing time on rewarding flowers during the course of the experiment, indicating an increased handling efficiency. Although it is in principle possible that our subjects reduced probing over the course of trials because of increased levels of satiation, it has been demonstrated in other studies that experienced pollinators can reduce their handling time substantially after only a few probing events, which greatly improves their foraging efficiency and intake rate (Laverty & Plowright 1988; Chittka et al. 1999; Raine & Chittka 2008) including Manduca sexta (Goyret & Raguso 2006).

In conclusion, we found evidence that the two observed potential partner control mechanisms in hawkmoths – reduced drinking duration and visit of fewer flowers on nectar-minimized plants – have a strong innate component, while learning seems to be of relatively minor importance in this context. This may in particular apply to potential early steps in the evolution of cheating, when individual plants offer less than the population average rather than no nectar at all. Avoidance learning of plants offering low nectar volumes rather than no nectar at all appears to be a difficult task: even minimal amounts of nectar as might have remained in our manipulated plants may stimulate the reward center in the brain (Kuwabara 1957). We hypothesize that the use of innate foraging strategies that simultaneously act as partner control mechanisms will strongly select against the evolution of cheating in plants, including species in which nectar production is costly. The next key step will be to test this hypothesis explicitly by measuring the fitness of individual plants that provide less nectar than the population average.

Acknowledgements

We thank Siobhan Braybrook for critically reading the manuscript, four anonymous reviewers for their valuable comments, Christopher Ball for plant care and the Swiss National Fund for financial support of this study.

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