Report

Hawkmoth Pollinators Decrease Seed Set of a Low-Nectar *Petunia axillaris* Line through Reduced Probing Time

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Summary

Although deception of floral pollinators is well known among orchids [1, 2], the majority of animal-pollinated plants secure pollination by nectar rewards. The costs and benefits of nectar production remain poorly understood [3–5]. Here, we developed a crossing design to introgress a low-nectar-volume locus of *Petunia integrifolia* into the genetic background of *P. axillaris*. The resulting introgression line resembled *P. axillaris* but produced only one-third of the nectar volume. When exposed simultaneously to low-nectar and wild-type *P. axillaris* plants, hawkmoth pollinators reduced their probing duration on low-nectar plants but otherwise did not show any signs of discrimination against these plants. However, reduced probing duration resulted in reduced seed production in the low-nectar plants despite their higher reproductive potential as evidenced by hand pollination. In line with this interpretation, we found a positive correlation between probing duration and seed set, and hawkmoth pollination of low-nectar plants that were manually supplemented with nectar to parental levels yielded seed sets similar to hand pollination. Thus, a simple self-serving pollinator behavior—the adjustment of probing time in response to nectar volume—may select against reducing nectar and protect many plant-pollinator mutualisms against a drift toward parasitism.

Results and Discussion

Mutualisms are cooperative interactions between individuals from two or more species in which all individuals gain a net benefit [6]. They appear in many biological systems and are believed to be critical in shaping nearly every existing ecosystem [7]. Most of these interactions involve investments (where cooperating leads to a reduction of the actor's immediate payoffs relative to individuals that do not cooperate) by at least one partner [6–9]. The existence of investments raises the question of which factors stabilize mutualisms and prevent cheaters from spreading in the population. To answer this question, one would ideally measure the fitness consequences of an individual with reduced investment relative to the wild-type. Here, we provide such data for a plant-pollinator mutualism involving *Petunia axillaris* ssp. *axillaris* N, a common laboratory strain of *P. axillaris*, and one of its main pollinators, the tobacco hornworm moth *Manduca sexta*, a nocturnal hawkmoth species.

Pollination mutualisms are usually asymmetrical interactions in the sense that only the plant invests in the production of a reward (typically floral nectar), whereas the pollinator ensures reproductive success of the plant by cross-fertilization as a by-product of self-serving foraging decisions. The vast majority of flowering plant species provide nectar rewards, suggesting that rewards are typically under positive selection (deceptive orchids being a well-known exception; for overview see [1, 2]). Nevertheless, there is disagreement concerning the actual costs and benefits of nectar production [10–12]. Optimal nectar volumes will depend strongly on pollinator behavior. Higher nectar volumes will make a plant more attractive, but such benefits must be balanced against the costs of production and the effects on flower visitation: if pollinators visit more flowers on the same plant and fewer plants overall, this will increase inbreeding and thus be under negative selection [13, 14].

Manipulation of nectar quantities has been instrumental in deducing pollinator decision rules with respect to nectar production that affect the fitness of plants [4, 15–17]. In addition, the reproductive success of deceptive versus nectar-providing orchid species has been compared [5, 18]. However, these approaches measure either the costs or the benefits of manual nectar manipulation. Here, we use a different approach in which net fitness consequences for individual plants with reduced nectar investment relative to conspecifics may be studied as a function of pollinator behavior. We exploited the interspecific fertility between two *Petunia* species, one with high nectar volumes (*P. axillaris* ssp. *axillaris* N, hereafter referred to as *P. axillaris*) and one producing low nectar volumes (*P. integrifolia* ssp. *inflata*, hereafter referred to as *P. integrifolia*). We introgressed a low-nectar locus from *P. integrifolia* into the genetic background of *P. axillaris* by repetitive backcrossing. First, we assessed all phenotypic traits known to affect pollinator behavior. Next, we studied the behavioral responses of tobacco hornworm moths to both our introgression line and wild-type plants. We were particularly interested in behaviors that may affect the fitness of a plant [3, 19], namely whether pollinators avoid low-nectar plants and whether they adjust probing duration in response to nectar volume. Finally, we tested whether probing duration may affect female reproductive success, measured as seed set (number of mature seeds per capsule) per flower after one pollination event.

Phenotypic Comparison of Wild-Type *P. axillaris* and Low-Nectar Introgression Line F25

Our aim was to produce an introgression line that would be similar to *P. axillaris* in all phenotypic traits except for nectar production. Our low-nectar introgression line (F25) largely fulfilled these criteria (Table 1; see also Figure S1 available online). F25 and control plants differed significantly in nectar volumes, with F25 containing on average only 30% of the nectar volume of *P. axillaris* (Table 1). The reduced nectar volume in F25 plants was accompanied by half the total nectar sugar compared to parental plants. Other phenotypic traits including flower anatomy and pollen/ovule production were similar, with the exception of methyl benzoate (MB) emission.

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Table 1. Comparison of Phenotypic Traits

	P. axillaris	F25	Comparison P. axillaris-F25	P. integrifolia	Comparison P. integrifolia-F25	Number of Measurements per Category
Tube length D1 (cm)	2.1 ± 0.1	2.1 ± 0.1	p = 0.35	0.2 ± 0.02	p < 0.001	10
Tube length D2 (cm)	1.2 ± 0.1	1.3 ± 0.1	p = 0.35	1.6 ± 0.2	p < 0.001	10
Corolla diameter (cm)	5.4 ± 0.3	5.6 ± 0.2	p = 0.28	3.1 ± 0.18	p < 0.001	10
Corolla UV reflectance	no	no		yes		1
Median MB emission (pptv)	18,156	52,836	p < 0.001	414	p < 0.001	17
Q_1	29,705	19,942		255		10
Q_3	59,148	141,665		440		
Number of pollen/flower	185,360 ± 12,990	196,289 ± 29,961	p = 0.78			10
Number of ovules/ovary	207.2 ± 30.6	196.2 ± 31	p = 0.485			6
Mean ovary weight (mg)	5.2 ± 0.6	5.3 ± 0.9	p = 0.766			17
Nectar volume (μl)	34.7 ± 6.8	10.4 ± 4.1	p < 0.001	1.35 ± 0.47	p < 0.001	10
Nectar sugar proportion (%)						2
Glucose	21	26		32		
Fructose	22	26		33		
Sucrose	57	48		35		
Nectar sugar concentration (%w/v)	16.53 ± 1.5	30.75 ± 4.3	p < 0.02	37.5	p = 0.262	6
Sugar (g/flower)	5.62 (100%)	3.17 (56%)		0.5 (9%)		6
Stigma surface area (cm²)	0.035	0.039	p = 0.243			10

Statistical analysis of average phenotypic parameters of *P. axillaris*, F25, and *P. integrifolia*. All values are given as averages ± standard deviation, except for methyl benzoate (MB) emission, given as median (pptv, particles per trillion volumetric). For an illustration of the similarities in morphological parameters between *P. axillaris* and F25, see Figure S1.

In F25, MB emission was nearly three times as high as in *P. axillaris* (Table 1). Consistent with our backcross design, the phenotype and genotype of our F25 line was much more like *P. axillaris* than its other parent, *P. integrifolia* (Figure S1; Table 1). For example, 65 genetic markers spread over the seven chromosomes of *Petunia* were homozygous for *P. axillaris*, whereas only one simple sequence repeat marker retained the *P. integrifolia* genotype.

Pollinator Behavior during Choice Experiments
We simultaneously exposed one *P. axillaris* and one low-nectar F25 plant to one hawkmoth at a time. We measured first choice, number of flowers visited per plant, and probing duration for each flower. There was no significant initial preference for either F25 (15 first approaches) or *P. axillaris* (21 first approaches) (n = 37, χ^2 = 0.7, df = 1, p = 0.4). Similarly, there was no difference in first choice when moths were offered F25 supplemented with 15 μl of nectar, bringing F25 up to *P. axillaris* nectar levels (7 first approaches) and *P. axillaris* (7 first approaches; n = 14).

The number of flowers visited was not significantly different between F25 (median 3 flowers) and control plants (median 3 flowers) (Wilcoxon signed-rank test; n = 25, Z = −1.37, p = 0.171) (Figure 1A). There was also no significant difference in the number of flowers visited between *P. axillaris* and F25 when the latter was supplemented with 15 μl of nectar (Wilcoxon signed-rank test; n = 14, Z = −0.184, p = 0.854). In this respect, our negative results differ from a previous study on *Petunia* in which hawkmoths visited fewer flowers on plants where nectar was manually removed [20].

Probing time per flower on F25 was significantly decreased compared to control plants (Wilcoxon signed-rank test; n = 25, Z = −4.39, p < 0.001). The mean probing time per flower on F25 was 6.93 s, compared to 11.14 s on *P. axillaris* (Figure 1B), representing a feeding time reduction of 47%. The difference in probing time was restored when F25 was supplemented with 15 μl of nectar (mean probing time 13.3 s) and tested against *P. axillaris* (mean probing time 14.3 s; Figure 1B) (Wilcoxon signed-rank test; n = 14, Z = −4.89, p = 0.625).

Taking these results together, we found no evidence for pollinator discrimination between the two plant lines. The only observed pollinator behavior that could potentially act as a control mechanism against reduced nectar production was probing duration. Therefore, next we analyzed how probing duration affected seed set in our two *Petunia* lines.

The Effects of Probing Duration on Seed Set
We compared seed set in wild-type and F25 plants both after hand pollination (as an indicator of maximum seed set [21]) and after one hawkmoth visit. There was a significant interaction between plant line and pollination method (Table S1). Post hoc tests revealed that hand-pollinated seed set was significantly higher (+20%) in F25 (n = 57, mean number of seeds 480 ± 20 SE) than in *P. axillaris* (n = 56, mean number of seeds 383 ± 20) (Tukey's honestly significant difference [HSD] test; p = 0.009). However, when both lines were pollinated by moths, F25 produced significantly fewer seeds (n = 52, mean number of seeds 328 ± 22) than *P. axillaris* (n = 40, mean number of seeds 424 ± 25) (−23%) (Tukey's HSD; p = 0.03). Additionally, moth-pollinated F25 plants showed a significant decrease in seed set compared to hand-pollinated F25 plants (−32%) (Tukey's HSD; p < 0.0001). The reproductive potential of moth-pollinated F25 plants was restored when nectar volumes were supplemented with 15 μl of nectar (n = 26, mean number of seeds 468 ± 30) (Tukey's HSD; p = 0.997) (Figure 2A). The number of seeds per flower was positively correlated with hawkmoth probing time on *P. axillaris* and F25 plants (n = 15, linear regression, R^2 = 0.336, p < 0.01; Figure 2B).

Game theoretic models propose that in cases in which payoffs are positively correlated with the duration of an interaction, an early ending in response to cheating by a partner may indeed be a suitable mechanism to diminish the payoffs for cheaters [22], as long as cheaters cannot easily find new partners. This condition is apparently fulfilled in our system because hawkmoth visits are low in wild *P. axillaris* populations [23], and several studies provide evidence that hawkmoth-pollinated plants are actually pollinator limited [24, 25].
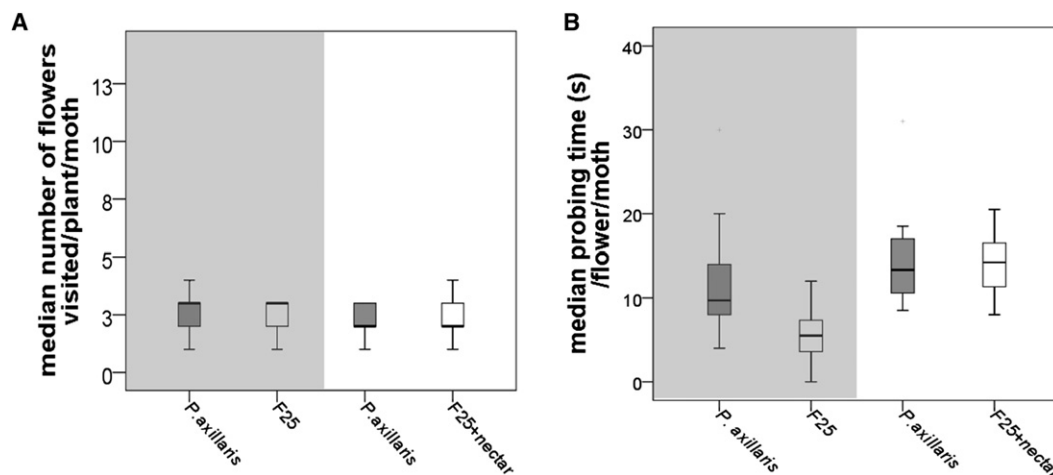
Figure 1. Hawkmoth Behavior on *P. axillaris*, Low-Nectar Line F25, and Refilled F25

(A) Left panel, highlighted in gray: box plots of number of flowers visited per plant and *M. sexta* flight in F25 compared to control *P. axillaris* plants. Right panel: number of flowers visited per plant and moth on *P. axillaris* plants and F25 supplemented with 15 μl nectar. The upper and lower limits of the box depict the first and third interquartiles; the thicker line is the median. Error bars depict the variance, with maximal value on top and minimal value on the bottom.

(B) Left panel, highlighted in gray: box plots of probing time per flower per hawkmoth on F25 compared to control *P. axillaris* plants. Right panel: probing time per flower per hawkmoth on *P. axillaris* plants and F25 supplemented with 15 μl nectar. The upper and lower limits of the box depict the first and third interquartiles; the middle line is the median. Error bars depict the variance, with maximal value on top and minimal value on the bottom.

Therefore, it is reasonable to assume that one pollinator visit per flower reflects natural conditions in our study system. Our results demonstrate that under such conditions, the reproductive potential of a flower is a function of pollinator probing duration, which in turn is a function of nectar volume. Thus, a very simple self-serving foraging rule of pollinators may select against a reduction of nectar volume in flowering plants. This mechanism is similar to host sanctions in legume-mycorrhiza interactions, where plants self-servingly grow roots in areas where nitrogen fixation is highest, which is typically where the most cooperative mycorrhiza lines live in the plant nodules [26, 27].

Previous studies on the relationship between nectar production and pollinator behavior have been limited to testing the effect of nectar depletion or addition on pollinator behavior. Although this approach is useful for testing changes in pollinator behavior or either costs or benefits, a simultaneous measurement of costs and benefits and hence fitness consequences cannot be assessed. In this context, our approach of employing introgression lines offers additional insights that cannot be easily obtained in other ways. Although the cost of nectar production is debated [10–12], if the cost is high, it follows that plants may benefit from reducing nectar production, if at all possible. In this context, two of our results are of interest. First, we found increased production in the F25 line of MB, a floral volatile compound known to attract hawkmoths [28–30]. Although there was no evidence in our experiments that increased MB emission had an effect on hawkmoth preference, our greenhouses were likely scent saturated, which would have masked any positive effects. Therefore, the potential benefits of increased scent production should be tested under field conditions. However, the chemical structure of MB is so different from sugar [31] that we currently consider it unlikely that there is a trade-off in the production of both substances. More generally, trade-offs with respect to the quantity of volatile productions are currently poorly understood (see for example [32, 33]). A second, more likely explanation for reallocating resources away from nectar production is increased seed set, a trait known to be enhanced by sugar [34]. We found evidence for increased potential seed set in the F25 line through hand pollination, while hawkmoth-pollinated wild-type plants showed an intermediate seed set. Again, field studies will be informative in this regard because natural light conditions will have a major influence on sugar production and, therefore, the potential trade-off between nectar production and seed set.

Conclusions

Understanding the causes of stable nectar production in flowering plants has been a major challenge. Much remains to be learned about the costs and benefits of nectar production in flowering plants. Our novel use of a low-nectar introgression line allows for comparative cost-benefit analysis of nectar production in a single species. We have demonstrated that reduced nectar production in *P. axillaris* causes reduced seed set under greenhouse conditions, and that this reduction is a function of decreased pollinator probing duration. Future experiments should be conducted under field conditions within the native range of *P. axillaris* and include measures of male reproductive success. From a game theoretical perspective, our results add to the growing awareness that, though complex partner control mechanisms may occur in mutualisms (for example, reputation-based access to partners [35]), simple self-serving mechanisms appear to be most widespread [6–8, 36, 37].

Experimental Procedures

The study was conducted at the Institute of Plant Sciences, University of Bern, from April 2007 through July 2011 using *Petunia axillaris* and an introgression line (F25) with reduced nectar volume but otherwise similar phenotype. For details on *P. axillaris*, growth conditions, and the breeding program resulting in a low-nectar line, see Supplemental Experimental Procedures.
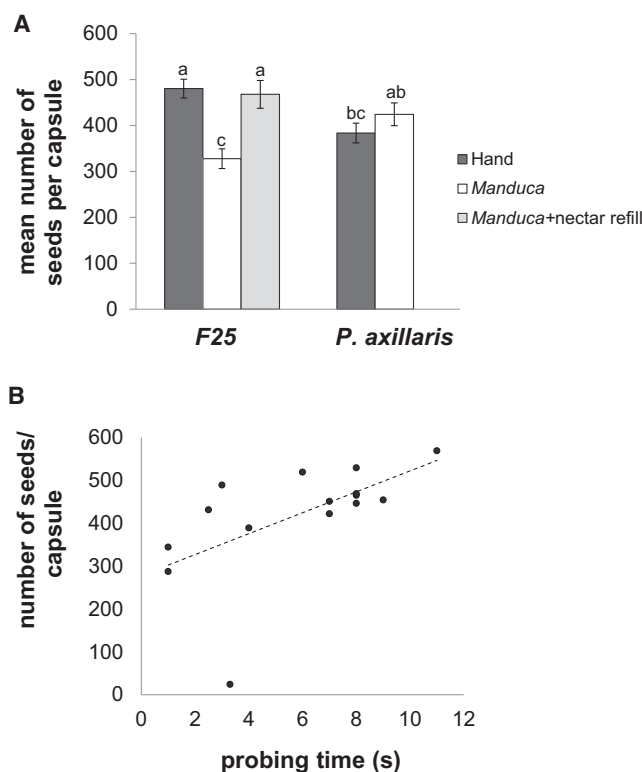
Figure 2. Seed Set of *P. axillaris* and Low-Nectar Line F25

(A) Comparison of mean number of seeds per capsule of low-nectar line F25 and *P. axillaris* with standard error, in both hand-pollinated and *M. sexta*-pollinated treatments. For statistical output underlying this figure, see Table S1.

(B) Linear regression model revealing positive relationship between hawkmoth probing time and number of seeds produced per flower.

Behavioral Experiments with Hawkmoth *Manduca sexta*

All behavioral experiments were conducted in a greenhouse featuring a flight arena and the pollinator species *Manduca sexta* (L.) (Sphingidae). We used vegetative propagations of both the low-nectar introgression line (F25) and *P. axillaris* to ensure a homogenous genotype. In hawkmoth behavioral assays, two plants were presented simultaneously, one *P. axillaris* and one F25 with either low nectar content or supplemented with 15 μl nectar. For details on insect care and handling and the experimental setup, see Supplemental Experimental Procedures.

Seed Set

We measured seed set capacities (number of seeds per capsule) as one parameter of female fitness of both F25 and *P. axillaris* plants using 15 vegetative propagations per category, obtained by hawkmoth pollination and by hand pollination. For details on the seed set experiments, see Supplemental Experimental Procedures.

Correlation of Seed Set to Probing Time

The experimental procedure was similar to seed set experiments, except that additionally, hawkmoth probing time per flower was measured and correlated to seed production. For additional information, see Supplemental Experimental Procedures.

For details on all statistical analyses, see Supplemental Experimental Procedures.

Supplemental Information

Supplemental Information includes one figure, one table, and Supplemental Experimental Procedures and can be found with this article online at http://dx.doi.org/10.1016/j.cub.2012.06.058.

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