
THE UNIVERSITY OF ARIZONA
University Libraries

UA CAMPUS
REPOSITORY

Nectar addition changes pollinator behavior but not plant reproduction in pollen□rewarding Lupinus argenteus

Item Type	Article
Authors	Heiling, Jacob M.; Bronstein, Judith L.; Irwin, Rebecca E.
Citation	Heiling, J. M., Bronstein, J. L., & Irwin, R. E. (2021). Nectar addition changes pollinator behavior but not plant reproduction in pollen□rewarding Lupinus argenteus. American Journal of Botany.
DOI	10.1002/ajb2.1613
Publisher	Wiley-Blackwell
Journal	American Journal of Botany
Rights	© 2021 Botanical Society of America
Download date	25/08/2021 17:17:58
Item License	http://onlinelibrary.wiley.com/termsAndConditions#vor
Version	Final accepted manuscript
Link to Item	http://hdl.handle.net/10150/657083

ORIGINAL ARTICLE

Nectar addition changes pollinator behavior but not plant reproduction in pollen-rewarding *Lupinus argenteus*

Jacob M. Heiling[1,2,3,], Judith L. Bronstein[2,4], and Rebecca E. Irwin[1,2]*

[1] Dept. of Applied Ecology, North Carolina State University, Raleigh, North Carolina, USA

[2] Rocky Mountain Biological Laboratory, Gothic, Colorado, USA

[3] Present Address: Dept. of Biological Science, Florida State University, Tallahassee, Florida, USA

[4] Dept. of Ecology and Evolutionary Biology, University of Arizona, Tucson, Arizona, USA

** For correspondence. E-mail address jheiling@fsu.edu*

Manuscript received _____; revision accepted _____.

Running title: Heiling et al. — Nectar addition in *Lupinus argenteus*

ABSTRACT

PREMISE: In addition to its role as the male gamete, pollen is often used as a food reward for pollinators. Roughly 20,000 species of angiosperms are strictly pollen-rewarding, providing no other rewards to their pollinators. However, the influence of this strategy on pollinator behavior and plant reproduction is poorly understood, especially relative to the nectar-reward strategy. We performed a field experiment using the strictly pollen-rewarding *Lupinus argenteus* to explore how the absence of nectar influences pollinator behavior and plant reproduction.

METHODS: We added artificial nectar to *L. argenteus* individuals to simulate a phenotype that would reward pollinators with both nectar and pollen. We compared bee pollinator behavior, via direct observation, and female reproduction between nectar-added and nectarless control plants.

KEY RESULTS: Bees exhibited behavioral responses to the novel reward, collecting nectar as well as pollen and spending 27% longer per flower. Pollen transfer increased with flower visit duration. However, plants in the study population were not pollen-limited; consequently, the observed changes in pollinator behavior did not result in changes in female components of plant reproduction.

CONCLUSIONS: The addition of nectar to pollen-rewarding plants resulted in modest increases in per-flower pollinator visit duration and pollen transfer, but had no effect on reproduction because, at the place and time the experiment was conducted, plants were

not pollen limited. These results suggest that a pollen-only reward strategy may allow plants that are visited by pollen foragers to minimize some costs of reproduction by eliminating investment in other rewards, such as nectar, without compromising female plant fitness.

Key Words: Bombus, foraging, floral reward strategy, pollen limitation, pollinator reward, nectar addition, novel phenotype, single-visit deposition

INTRODUCTION

Nectar is likely the most widespread reward that flowering plants provide to pollinators, but it is not the ancestral condition in angiosperms (Armbruster, 2012). Plant gametophytes (pollen and ovules) are thought to have been among the first floral resources for which insects visited flowers and were the first floral rewards (Simpson and Neff, 1983; Luo et al., 2018). Many angiosperm species offer both pollen and nectar rewards, other species are strictly nectar-rewarding, while others have maintained or secondarily derived a strictly pollen-reward strategy. In some cases, all three of these strategies can be found within a plant family (e.g., Papaveraceae, Solanaceae, Orobanchaceae, and Fabaceae; Simpson and Neff, 1983; Macior et al., 2001; Etcheverry et al., 2012).

The use of pollen as the sole reward represents a paradox: pollen-rewarding plants pay for the movement of gametes with gametes. Every grain of pollen consumed by a pollinator is a lost reproductive opportunity (Armbruster, 2012). Strictly pollen-rewarding plants often make more pollen per flower than do close relatives that offer nectar and pollen (Etcheverry et al., 2012, but see Cruden, 2000 for a comparison across broad plant families), presumably to offset lost reproductive opportunities due to pollen consumption (Darwin, 1859). Why should a plant use its gametes as a reward? Inroads to addressing this question may be made by first asking more proximate questions about pollen rewards. In particular, comparing the pollen-reward strategy to other strategies may inform our understanding of its ecological consequences for plant reproduction. Here we ask, how might offering nectar affect pollinator behavior and plant fitness in pollen-rewarding plant species? A better understanding of how the

different reward strategies mediate pollinator behavior, and how reward-mediated variation in pollinator behavior influences plant reproduction, are first steps in understanding why some lineages have maintained or re-derived this character state while others (often close relatives) have not.

Here we explore whether benefits to plants would differ between the pollen-only and the pollen + nectar reward strategies, and whether such differences could be explained by pollinator foraging behavior. We did so by comparing the behavior of pollinators and components of reproduction in the strictly pollen rewarding species silvery lupine (*Lupinus argenteus* Pursh, Fabaceae) to individual plants whose flowers were augmented with artificial nectar. While bees engaged in pollen foraging are primarily seeking pollen rewards, pollen foragers are known to also collect nectar during pollen foraging trips (Heinrich, 1976). Thus, *L. argenteus* individuals bearing both pollen and novel nectar rewards effectively represent richer resource patches for foraging bees than natural, nectarless, plants. As a result, we predicted an increase in pollinator visitation (i.e., more frequent or longer foraging at more rewarding flowers or plants, from the perspective of the bee), either via increased numbers of visits, increased time spent per flower, or both, in nectar-added plants. This prediction follows from the Marginal Value Theorem (Charnov, 1976), which predicts, in part, that foragers will spend longer in patches with higher than average resource availability, a prediction that has been supported in several experimental and observational pollinator foraging studies (e.g., Pyke, 1978; Thomson, 1986). If such increased visitation results in increased pollen deposition, and if seed set is pollen-limited, we predicted an increase in female reproductive success in nectar-added plants relative to controls. Importantly,

while increased visit frequency and duration often result in increased pollen deposition, this relationship cannot be assumed for all systems (King et al., 2013), and must be assessed empirically. Specifically, we asked four questions. (1) Will pollinators discover and collect nectar if it is added to the flowers of a species in which they have not previously encountered nectar? Finding that bee pollinators do feed on added nectar, we asked: (2) How does nectar addition affect pollinator foraging behavior and plant reproductive success? Finding a significant positive effect of nectar addition on flower visit duration but not on female reproductive success, we then asked: (3) How does time spent per flower affect single-visit pollen deposition in *L. argenteus*, and (4) Does *L. argenteus* exhibit pollen limitation?

We note that while the mating cost imposed by pollen consumption is a cost to reproductive success through male function, benefits associated with the pollen-reward strategy may come through male function, female function, or both (Stanton et al., 1986; Wilson et al., 1994). Due to the difficulty of reliably assessing reproductive success through male function in the field (Snow and Lewis, 1993), and the unique difficulty of accessing or manipulating the androecium of *Lupinus* species without destroying other floral structures, in this study we focused our direct measurements of plant reproduction through female components alone. However, because movement patterns of pollinators can predict patterns of male mating success (Klinkhamer et al., 1994; Harder and Barrett, 1995; Stanton et al., 1986), our assessments of pollinator behaviors on nectar-added and control plants can serve as indirect proxies for variation in male mating success.

MATERIALS AND METHODS

Study system—

Lupinus argenteus is a long-lived herbaceous perennial, occurring in dry meadows and rocky slopes from the montane to alpine zones across the western United States and into northern Mexico. We studied *L. argenteus* in the Gunnison National Forest in the Elk Mountains of Colorado, USA near the Rocky Mountain Biological Laboratory (RMBL). The papilionaceous flowers are blue to purple with white banner spots and are borne in whorls on indeterminate stalks. Plants produce 1–40+ flowering stalks at a time (median 4 stalks per plant) with 1–20 flowers per stalk (median 6 flowers per stalk). Flowers are receptive and pollen is viable for approx. 5 d, remaining on the stalk (following color change of the banner petal from white to purple) for an additional 5-7 d (Gori, 1989). A single plant produces a few to nearly 1000 flowers in a season (mean = 300 flowers/season at our site). As with other lupines (Dunn, 1956; Müller, 1873), *L. argenteus* is strictly pollen-rewarding (Gori, 1989); flowers are invariably dry when dissected and staining flowers with neutral red reveals no floral nectaries (J. Heiling *pers. obs.*). *Lupinus argenteus* is self-incompatible and pollinated by pollen-foraging bees, primarily in the genera *Bombus*, *Andrena*, and *Megachile* (Dunn, 1956; Gori, 1989; Harder, 1990). Pollen is dispensed from a pore in the distal end of semi-fused keel petals (Müller, 1873) which must be depressed away from the axis formed by the banner and petiole to eject pollen. A fixed volume of pollen is dispensed at each visit (Harder, 1990), with the keel refilling with pollen as anthers dehisce following removal. Bees activate the piston-like mechanism by bracing their heads on the banner petal (Fig. 1a and Fig. 2b), clutching the wing petals (Fig. 1a.2) that enclose the keel (Fig. 1a) with their mid- or forelegs (Fig. 2b), and then extending

their legs (Fig. 2c), resulting in deposition of pollen on their abdomen or thorax (Dunn, 1956). Bees typically approach lupine flowers with their glossas extended (Fig. 2a) and probe the inner corolla space with their glossas while positioning themselves (Fig. 2b). Despite the absence of nectar rewards in natural lupine corollas, the corolla probing behavior observed in lupines has been interpreted as attempted nectar seeking (Heinrich, 1976); nectar accumulation occurs in this inner corolla space in many nectar-producing papilionaceous flowers (e.g., *Vicia* spp.; Stpiczyńska, 1995). Alternatively, the use of the glossa in this way may facilitate landing and positioning (e.g., Fig 2a). Regardless of the cause of this behavior, it presented the opportunity to place artificial nectar in a location that pollen foraging bees would be likely to discover it. Finally, while collecting pollen from lupine flowers, bees' bodies do not come in contact with the area between the wing and keel petals (Fig. 1b.5), with their abdomen or thorax (depending on body size) only contacting the tip of the keel (Fig. 1b.4) while operating the piston mechanism (Fig. 2c). As a result, the inner corolla area (between the wing and keel petals) is an ideal location in which to deposit artificial nectar (Fig. 1b.6).

Do pollinators respond to nectar addition by removing nectar?—

To assess whether bees discover and collect artificial nectar in lupine flowers, we added 2 µl of artificial nectar (hereafter, referred to simply as nectar; 40% v/v sucrose, concentration chosen to mimic the co-flowering community, Luo et al., 2014) to all open flowers on three *L. argenteus* individuals (one individual per d on 25-27 July 2017) at our study site (Bellview Bench, N: 39.00490°, W: -107.03182°, 3,139 masl, approx. 11×11 m). We deposited nectar between the keel and wing petals (Fig. 1a&b) using a micropipette (Fisherbrand, HandyStep, Thermo Fisher Scientific, Waltham,

Massachusetts, USA). We then directly observed the stalks. When a bee initiated a visit to a stalk, we allowed her to visit several flowers, but shooed her away before she revisited any flowers or visited all flowers on the stalk. We then used 5 µL microcapillary tubes to measure the residual nectar in each visited flower and in a neighboring unvisited flower on the same stalk. Once a stalk was visited, we removed it from the plant.

We used a paired t-test to compare the within-stalk mean nectar volumes of visited and unvisited flowers. We conducted the analysis on nectar volume means per stalk for visited vs. unvisited flowers to prevent pseudoreplication. R version 3.5.1 (R Core Team, 2018) was used for all statistical analyses.

How does nectar addition affect pollinator foraging behavior and plant reproductive success?—

We arbitrarily selected 98 *L. argenteus* individuals at Bellview Bench (the majority of *L. argenteus* at the site) and randomly assigned them to one of two treatments: nectar addition (pollen + nectar rewarding plants, N = 49) or control (pollen-rewarding only, N = 49). Daily nectar addition to all open flowers on nectar-added plants (with order randomized daily) began soon after flowering (on 14 July 2016) and was discontinued when plants passed peak bloom (13 Aug 2016). Floral display size (open flowers per plant) was recorded on all plants each day of nectar addition. We probed flowers on control plants with a dry micropipette tip to control for flower handling. On both the nectar-added and control plants, we cleaned the micropipette tip with ethanol between plants to prevent pollen transfer. To avoid diluting the added nectar with morning dew, we added it once plants were dry, approx. 10:00-11:00AM.

We directly observed bee activity for 14 d between 19 July and 4 Aug 2016. Observations were conducted for 1 h per d immediately following nectar addition; pilot trials using unvisited flowers (protected with mesh bags to prevent bee visitation) showed that nectar volume decreased measurably in field conditions after that period of time, likely due to evaporation. All observations were documented using a digital voice recorder. When we spotted a bee visiting an experimental plant, we followed her and recorded her behavior until she left the study area or flew out of sight. We recorded (1) the approaches to a plant that resulted in flower visitation, summed across the flowering season, to each individual plant (hereafter “total visits”), (2) the number of flowers probed per plant per plant visit, and (3) the time spent per flower (in seconds). We also recorded the species and caste of each visitor; only females (workers and queens) were observed visiting *L. argenteus*. Each time a bout was observed, the bee received a unique ID in our dataset. Because we did not mark bees, it was not possible to determine whether a bee had been observed previously and so we were unable to analyze behavior at the level of the individual bee. Accordingly, we treated visitor ID as a random effect in all analyses.

We did not bag experimental stalks following observations. While it is possible that subsequent visits to experimental plants following the removal or evaporation of nectar may have reduced the overall signal of the nectar treatment on female reproductive success, we chose to leave stalks unbagged in order to facilitate a more biologically realistic design, in which plants remained available to visitors regardless of their reward status.

In order to assess the effect of nectar addition on female reproductive success, we collected all fruit-bearing stalks from nectar addition and control plants between 29 Aug and 4 Sept 2016, allowing fruits to dry on the stalk before dissecting them to count the seeds in each fruit. We weighed the seeds per plant to the nearest 0.1 mg.

We used the following packages in R to statistically analyze the data. We used *stats* to fit linear models (LM), *MASS* to fit generalized linear models (GLM) (Venables and Ripley, 2002), *lme4* to fit linear and generalized mixed effects models (LMM and GLMM) (Bates, 2010), and *'lmerTest'* (Kuznetsova et al. 2015) and *'piecewiseSEM'* (Lefcheck, 2016) to perform inference tests and to estimate pseudo r-squares for GLMMs, respectively.

To understand how nectar addition affected pollinator behavior, we analyzed (1) total visits per plant, (2) number of flowers probed per plant visit, and (3) time spent per flower. We assessed the effect of nectar treatment on total visits using a generalized linear model with a negative binomial error distribution (data were overdispersed, dispersion parameter = 2.04; θ = 1.602, maximum likelihood) with nectar treatment as the main predictor and mean floral display (mean open flowers per individual per d across the season) as a covariate. To assess the effect of nectar treatment on the number of flowers probed per visit, we fit a Poisson GLMM with nectar treatment and floral display size (on the day a visit was observed) as fixed effects. Random effects included visitor ID (to account for multiple observations of a single individual bee), plant ID (to account for resampling of individual plants within d and across the season) and date nested within plant. We scaled display size using the base R function *scale()* to reduce Eigenvalue size and improve model fit. We compared the time visitors spent per

flower on nectar addition and control plants using natural log transformed mean flower visit duration (transformed to meet assumptions of Gaussian distribution) as the response with nectar treatment (addition vs. control) and display size (scaled as above) as fixed effects in a LMM, with visitor ID and date nested within individual plant as random effects.

We measured three components of female reproductive success: (1) total seed production (all mature seeds per plant), (2) mean seed set per fruit per plant, and (3) mean weight per seed per plant. Because we did not mark individual flowers, and because *L. argenteus* fruits do not leave scars on the stalk, we could not calculate proportion fruit set. To assess the effect of nectar treatment (addition vs. control) on total seed production per plant, we fit a negative binomial GLM (seed count data were overdispersed, $\theta = 0.765$, maximum likelihood) with total seed production and mean floral display size as fixed effects. Seeds per fruit (Shapiro–Wilk: $W = 0.920$, $P < 0.0001$) and mean seed mass ($W = 0.946$, $P = 0.003$) were not normally distributed. Accordingly, we assessed the effects of nectar treatment on the mean number of seeds per fruit per plant and mean mass of mature seeds using two-sample Wilcoxon tests (R function *wilcox.test()* in the *'stats'* package).

How does time spent per flower affect single-visit pollen deposition?—

To determine whether pollinator effectiveness (measured as single-visit pollen deposition on a stigma) changed as a function of the length of a pollinator visit, we used the interview-stick method (Thomson, 1981). We affixed unvisited *L. argenteus* inflorescences to the end of a 1 m rod, and treated the flowers with either 0, 2, or 4 µL of nectar. We used a range of nectar volumes to promote variation in visit duration, not

to test for nectar volume preference among visitors. Inflorescences on interview sticks (Thomson, 1981) were offered to bees observed foraging on *L. argenteus* in our site. When a bee accepted a flower, we recorded the duration of the visit using digital voice recorders, shooing the bee away when she exited the first flower that she accepted. We then removed the stigma from that flower with forceps and stored it in a vial on ice. In the lab on the same day, we mounted stigmas on glass microscope slides with basic fuchsin gel (Kearns and Inouye, 1993) and counted the number of conspecific and heterospecific pollen grains at 400 × under a compound microscope (N = 24 stigmas). We ran interview-stick trials on three d between 28 July and 12 August 2017. Heterospecific pollen deposition was numerically low (< three grains found on three stigmas) and was not include in our analyses.

To assess the effect of visit duration on single-visit pollen deposition, we fit a negative binomial GLMM (pollen count data were overdispersed, dispersion parameter = 2.62; θ = 3.098, maximum likelihood) with visit duration, nectar treatment (2 and 4 µL nectar addition and control) and their interaction as fixed effects, and plant ID included as a random effect to account for sampling multiple stigmas within interview sticks. To quantify the effect of nectar volume on visit duration, we also fit a LMM with visit duration as the response and nectar volume (0, 2, or 4 µL) as a fixed effect and interview stick ID as a random effect.

Is* L. argenteus *reproduction pollen-limited?—

To evaluate whether seed set in the *L. argenteus* population at Bellview Bench was pollen-limited, we randomly assigned 25 individual plants to a pollen supplementation treatment and another 25 to a control group on 7 July 2017. For plants

in the pollen supplementation treatment, beginning at the onset of flowering and continuing until senescence (11 July-17 Aug 2017), we hand-pollinated all open flowers every third day during the approx. 96-h period of stigma receptivity (Gori, 1989). We used pollen mixed from five individuals (10-20 flowers each) which were situated 10-20 m away from experimental plants to reduce the likelihood of both outbreeding depression and bi-parental inbreeding (Waser and Williams, 2001). To ensure pollen viability, we limited pollen collection to flowers with white banner spots from the terminal or sub-terminal whorl in anthesis; pollen viability declines in *L. argenteus* after approx. 48 h post-anthesis (Gori, 1989). To apply pollen to stigmas, we used two pairs of forceps to gently pull back the banner and wing petals, exposing the stigma, and applied pollen to the stigma with a camel hair paintbrush (Kearns and Inouye, 1993). This pollen addition left a visible dab of pollen on the stigma. Hand-pollination using similar methods in other plant species results in increased pollen addition to flowers relative to open-pollinated control flowers (Kearns and Inouye, 1993). We counted each open flower on each plant and, using an oil-based paint pen (Sharpie, Oak Brook, Illinois, USA), applied a dot of blue paint to the adaxial surface of the calyx to prevent re-treating or re-counting flowers on subsequent days. To control for the effects of handling, we manipulated the flowers of control plants with forceps during counting.

At the end of the flowering period, we enclosed all stalks in bridal veil bags to prevent dehiscent seed loss and frugivory by deer and marmots during fruit ripening. We harvested plants when all of their fruit were ripe (dry and brittle), and counted all seeds per fruit per plant, weighing seeds to the nearest 0.1 mg. We recorded (1) proportion fruit set (number of seed-bearing fruits divided by the total flowers per plant);

(2) total seeds per plant, (3) mean number of seeds per seed-bearing fruit, and (4) mean seed mass.

To assess the effect of hand pollination on female reproductive success, we fit linear models with treatment (hand pollination vs. control) as our predictor and with total number of flowers (to test for effects of plant size or resources) as a covariate. The covariate was not significant in any model and was dropped. This model passed Durban-Watson and studentized Breusch-Pagan tests for residual autocorrelation and heteroskedasticity, respectively. Seeds per fruit were not normally distributed (Shapiro-Wilk: $W = 0.907$, $P = 0.004$). Accordingly, we assessed the effects of supplemental hand-pollination on the mean numbers of seeds per fruit per plant using two-sample Wilcoxon rank sum tests. We used a t-test to assess the effects of hand-pollination on mean seed mass.

RESULTS

Do pollinators respond to nectar addition by removing nectar?—

We observed 21 total visits by 9 individual bees (five *Bombus bifarius*, two *Megachile* sp., one *B. californicus*, and one *B. flavifrons*) to nectar-added stalks in the nectar-removal experiment. Bees collected both nectar and pollen during these visits. Bees were directly observed inserting their glossas into the space between the wing and keel petals where we had deposited the nectar droplet, then pulsating their abdomens, consistent with nectar consumption. Visited flowers had 50% less added

nectar compared to unvisited flowers (paired t-test: $t_8 = -4.89$, P = 0.001; Fig. 3). Moreover, we recovered none of the added nectar from 11 of the 21 visited flowers.

How does nectar addition affect pollinator foraging behavior and plant reproductive success?—

Over the 2016 season, we manipulated a total of 86,900 flowers (nectar added: 45,746 flowers; control: 41,154 flowers) on the 94 focal plants that flowered (nectar added: N = 45 plants; control: N = 49 plants). We observed 170 bee approaches that resulted in visits to focal plants, totaling 2,947 individual flower visits, across >14 h of observation. We observed eight bee taxa foraging on the focal plants, five identified to species (*Bombus appositus*, *B. bifarius*, *B. californicus*, *B. flavifrons*, and *B. mixtus*) and three identified to genus (*Andrena* sp., *Megachile* sp., and *Osmia* sp.). The most common visitors were *B. bifarius*, *B. flavifrons* (both ~ 2.89 mm intertegular distance; Pardee et al. *unpublished*), and a similarly sized *Megachile* sp. Together, these three taxa accounted for 93% of all observed visits (51%, 35%, and 7% of visits, respectively). *Bombus* visitors were all females, predominantly workers, with one queen *B. bifarius* and one queen *B. flavifrons* (out of 57 and 42 bees, respectively). All bee species responded similarly to nectar added and control plants, with no significant differences in behavioral responses between species (analyses not shown). As a result, we present results of models on pollinator foraging behavior in which all of the bee species were pooled.

Bees spent 27% longer per flower on nectar-added than on control plants ($t_{40.27} = 3.832$, P < 0.001; Fig. 4). However, neither total visits (control: 1.510 ± 0.302 (mean ± SE) visits per plant, nectar-added: 1.979 ± 0.313 visits per plant; $t_{95} = 1.152$, P = 0.252)

nor number of flowers probed per visit (control: 18.73 ± 2.69 flowers per visit, nectar-added: 16.12 ± 1.65 flowers per visit; Z = 1.100, P = 0.271) differed between treatments. There was a significant positive effect of the covariate mean floral display size on the total number of times plants were visited (Estimate = 0.024 ± 0.003 SE, t_{95} = 8.078, P < 0.0001) and on the number of flowers probed per plant visit (Estimate = 0.301 ± 0.085 SE, Z = 3.569, P = 0.0004). However, there was no effect of mean floral display size on time spent per flower ($t_{54.11}$ = 1.824, P = 0.074).

Although time spent per flower increased with nectar addition, we found no effect of nectar addition on any metric of plant reproduction, including the number of seeds per plant (Z = -0.854, P = 0.393), number of seeds per fruit (Z = -1.3729, P = 0.085), or mean seed mass (Z = -0.515, P = 0.303; Appendix S1, see the Supplementary Data with this article). Mean floral display size had a significant positive effect on total seed production (Estimate = 0.028 ± 0.004, Z = 7.465, P < 0.0001; Appendix S2).

How does time spent per flower affect single-visit pollen deposition?—

Flower visit duration had a significant positive effect on single-visit pollen deposition (Estimate = 0.178 ± 0.051 SE; conditional R^2 = 0.616; Z = 3.493, P < 0.0005; Fig. 5). However, nectar volume (0, 2, or 4 µL per-flower) did not have a significant effect on visit duration in these trials (Z = 0.238, P = 0.812).

Is* L. argenteus *reproduction pollen-limited?—

We found no evidence that reproduction in the study population was pollen-limited. There was no effect of supplemental hand pollination relative to open-pollinated controls on proportion fruit set ($F_{1,37}$ = 1.240, P = 0.273), total seed production ($F_{1,37}$ =

0.102, P = 0.752), mean seed production per fruit (Z = 0.107, P = 0.543), or mean seed mass ($t_{30.4}$ = -0.671, P = 0.507; Appendix S3).

DISCUSSION

Rewards are the foundation of resource-service mutualisms, and floral rewards are the primary ecological link between pollinator behavior and plant fitness (Parachnowitsch et al., 2018). The existence of resource-service mutualisms depends on the ability of the partner that provides resources to balance its production costs against the benefit it derives from interacting with the consumer (Bronstein, 2001). However, we know little about the selection pressures responsible for driving which floral reward strategies plant species employ, the fitness trade-offs associated with alternative reward strategies, or the feedbacks between floral reward strategies and pollinator behaviors. Here, we have shown that providing pollen as a sole floral reward allows *L. argenteus* to secure sufficient pollination service from pollen-collecting bees, and that an alternative pollen + nectar reward strategy may not change the frequency or outcome of the interactions between this species and its pollinators.

Visitors responded positively to nectar addition by lengthening their visits to flowers, and single-visit pollen deposition to stigmas increased modestly but significantly with flower visit duration. In contrast, nectar addition did not affect the total number of visits plants received or the number of flowers probed per plant visit.

Although nectar addition increased per-flower visit duration and pollen deposition, these effects did not translate into differences in female components of plant

389 reproduction, as would have been expected if plants were pollen limited (Thøstesen and
390 Olesen, 1996). It is therefore likely that, in this population, pollen deposition does not
391 limit reproduction. However, pollen limitation can vary in space and time (Campbell,
392 1987; Price et al., 2005). In sites or years when pollinator visits are rarer (due to
393 variation in pollinator demography or competition with co-flowering species), increased
394 visit duration, such as what was seen when nectar was added to flowers in this study,
395 could potentially alleviate pollen limitation. While the effect of visit duration on pollen
396 deposition was modest, small differences in pollen deposition could mean the difference
397 between full seed-set and a failure to reproduce for lupines, which bear < 10 ovules per
398 ovary (e.g., Wainwright, 1978). Thus, the absence of nectar production and the use of
399 pollen as a sole pollinator reward in lupines appears to be a reward mode whose
400 effectiveness may depend heavily on temporal and spatial variation in pollinator
401 availability.

402 The strictly pollen rewarding state may favor the cost savings (i.e., of carbon and
403 water) over reproductive assurance in any given season. One benefit of experimental
404 nectar addition is that it allows assessment of the pollinator-mediated effects of nectar
405 presence on plant reproduction independent of potentially confounding effects of
406 variation in resource investment. For long-lived plants that occur in xeric and/or highly
407 variable environments, such as lupines occurring in the montane to alpine zones of the
408 Western United States, year-to-year survival may pose a greater limit to lifetime fitness
409 than the relative success of any single reproductive bout (Bell and Bliss, 1980; Morris
410 and Doak, 1998). Further, water limitation results in limitation of nitrogen fixation in *L.*
411 *argenteus* (Goergen et al., 2009), potentially compounding the total cost of water

relative to nitrogen. Pollen is a relatively nitrogen-rich tissue (Rabie et al., 1983), and nitrogen itself, while typically limiting, may bear a relatively lower cost than water to nitrogen fixers growing in xeric soils. As a result, a water-saving reward strategy (such as providing pollen rather than nectar rewards) could come with substantial lifetime fitness benefits via increased long-term survival and higher nitrogen fixation rates (Goergen et al., 2009), especially if nectar were costly to produce in lupine, a point which requires further examination.

Another possible, and not mutually exclusive, benefit of the strictly pollen rewarding character state may be the filtering of the pollinator community (fewer groups of pollinators forage for pollen than nectar). Limiting rewards to pollen would reduce visitation by nectar-foraging visitors, potentially reducing competition and heterospecific pollen transfer (e.g., Muchhala, et al., 2010). The very low numbers of heterospecific pollen grains found on the stigmas that we sampled (3 grains total) is consistent with this hypothesis. However, pollinator filtering may be costly if it limits pollinator availability, and may leave plants especially vulnerable to pollinator loss. Thus, whether such filtering would be beneficial or costly is highly context dependent.

The only other experimental field study of which we are aware that compares the effects of nectar only and nectar + pollen reward strategies in a pollen-rewarding species was performed in the Himalayas by Tong et al. (2018). Contrary to the results presented here, Tong et al. (2018) describe negative effects of nectar addition on plant reproduction in the pollen-rewarding *Pedicularis dichotoma* (Orobanchaceae). They suggest that these effects are the result of altered body positioning and floral handling in bee pollinators foraging on nectar-added and nectarless inflorescences owing to the

asymmetrical floral architecture of this species. Conversely, in our study, the handling behavior of pollinators was consistent both within and among bee species (and consistent with observations of others, e.g., Dunn, 1956; Harder, 1990) on the bilaterally symmetrical flowers of *L. argenteus* regardless of the nectar status of flowers. Further, Tong et al. (2018) described an increased visitation frequency to nectar-supplemented flowers, an effect that was absent in *L. argenteus*. We offer two hypotheses to explain this difference. First, community-level floral reward availability varies substantially across communities (Ornelas et al., 2007; Ogilvie and Forrest, 2017) and as a function of soil moisture (Waser and Price, 2016). Subsequently, pollinator behavior varies in response to resource availability (Pleasants ,1981; Irwin and Maloof, 2002). If overall nectar availability were lower in the co-flowering community of *P. dichotoma* compared to that of *L. argenteus*, then artificial nectar may hold a higher relative value to bees in the *P. dichotoma* system. This hypothesis could be evaluated through simple comparative studies of the nectar availability and production rates among the nectar-producing species in each community (e.g., Luo et al., 2014) paired with experimental nectar addition across a range of wetter and drier sites or years. Second, even closely related and co-occurring bee taxa vary widely in their foraging behaviors (e.g., Irwin and Maloof, 2002) and their responses to resource availability (e.g., Ogilvie and Forrest, 2017). Thus, differences in foraging behavior between the pollinators of *P. dichotoma* (*Bombus friseanus*) (Tong et al., 2018) and *L. argenteus* (primarily *B. bifarius*) may drive differences in the outcomes of nectar addition. More studies are needed to understand whether or not there are general patterns associated with the effects of nectar addition on pollinator behavior and plant reproduction in strictly pollen rewarding species.

Although this study focused on measures of plant reproduction via female function, we can use the movement patterns and foraging behaviors that we observed to offer hypotheses regarding patterns of male mating success in response to nectar addition (Klinkhamer et al., 1994; Harder and Barrett 1995; Stanton et al., 1986). Generally, increased flower visit duration could affect male function independently of female function (e.g., Golubov et al., 1999) in two ways. First, pollen removal may increase with visit duration (Harder, 1990; Kudo, 2003), possibly reducing pollen export efficiency due to increased proportional pollen loss (Klinkhamer et al., 1994; Thomson et al., 2000). However, *Lupinus* species characteristically employ dynamic pollen release (i.e., variable quantities of pollen released per visit) as a function of visitation frequency (Harder and Thomson, 1989). Thus, increased visit duration may have little effect on pollen removal even though it did have a significant effect on pollen deposition. Second, when there is a positive relationship between flower visit duration and single-visit deposition, increased visit duration might increase pollen discounting through geitonogamy (de Jong et al., 1993), resulting in decreased male mating success. Generally, individuals with larger floral displays are expected to export more pollen (Queller, 1997; but see Wilson et al., 1994), but they may also suffer higher pollen discounting (Klinkhamer et al., 1994). When such a tradeoff exists, a phenotype that provides larger overall rewards (such as the novel nectar and pollen-rewarding phenotype that we have simulated here) may compound this effect and depress male mating success in larger plants. Conversely, individuals with smaller displays are generally less attractive and may struggle to export their pollen (Klinkhamer and de Jong, 1993). If the presence of nectar could reduce the threshold display size required

to compete for pollinators, then it may result in increased pollen export in smaller plants. Thus, the potential influence of a nectar reward in this species on reproductive success through male function might vary with plant size, and may be expected to either magnify (in the case of larger plants) or mitigate (in the case of smaller plants) negative effects of plant size on successful pollen export.

CONCLUSIONS

The evolutionary trajectories of reward strategies should be shaped by the unique combination of limitations and opportunities experienced by a plant lineage and, in turn, determine the species with which they can form and maintain mutualistic interactions. It is likely that pollen-rewarding species evolve in lineages that are able to balance the costs of pollen loss against the benefits of pollen transport better than those that provide other rewards (or no reward at all). If resource and reproductive opportunity costs of consumptive pollen loss are balanced against effective and reliable pollination by pollen foragers, as observed in *L. argenteus*, then there may be nothing to gain from providing alternative rewards such as nectar. Thus, a strictly pollen-rewarding strategy may be the outcome of accepting some opportunity cost (lost pollen) while minimizing other reward allocation costs, without compromising the benefit provided by pollinator visitation.

ACKNOWLEDGEMENTS

The authors thank D. Jacobsmeyer, K. Brennan, J. Ogilvie, M. Stemkovski for field and lab assistance; M. Stemkovski and G. Calabrese for statistical advice; I. Hennes for image editing expertise; J. Thomson for advice and conversations, and J. Thomson, W.

Morris, K. Gross, N. Haddad, C. Goodwillie, and two anonymous reviewers for comments on the manuscript. Access to field sites was provided by the RMBL and the USFS. We acknowledge that we performed our field work on traditional Parianuches (Ute) territory. This work was funded by the Colorado Mountain Club Foundation, RMBL, NC State University, and the National Science Foundation to R.I. (DEB-1641243). Any opinions, findings and conclusions or recommendations expressed in this material are those of the authors and do not necessarily reflect the views of the National Science Foundation.

AUTHOR CONTRIBUTIONS

All authors designed the study and acquired funding. J.H. conducted the investigation, data curation, visualization, and wrote the original draft with input and resources provided by J.B. and R.I. J.H. and R.I. performed the formal analyses. All authors reviewed and edited the manuscript.

DATA AVAILABILITY

All data associated with this manuscript are archived with the Open Science Framework (https://osf.io). DOI: 10.17605/OSF.IO/4AWPT

SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information section at the end of the article and include: Appendix S1: Means and SE of *L. argenteus* reproduction metrics from the nectar addition experiment. Appendix S2: Means and SE of *L. argenteus* reproduction metrics pollen-limitation experiment. S3: Total seeds per individual in the nectar addition experiment. Solid line: fitted curve for

nectar-added plants (pollen + nectar); dashed line: fitted curve for control plants (pollen only). Circles and triangles are raw data points. Bands are 95% CI.

LITERATURE CITED

Armbruster, W.S. 2012. Evolution and ecological implications of “specialized” pollinator rewards. In: Patiny S, ed. *Evolution of Plant-Pollinator Relationships*. Cambridge: Cambridge University Press, 44-67.

Bates, D., Maechler, M., Bolker B., Walker S., Christensen R.H.B., Singmann H., Dai B., and Grothendieck, G. 2014. Package lme4. R Foundation for Statistical Computing, Vienna, Austria. Website: https ://cran.r-proje ct.org/web/packages/lme4/index.html.

Bronstein, J.L. 2001. The costs of mutualism. *American Zoologist* 41: 825–839.

Bell, K. L., & Bliss, L. C. 1980. Plant reproduction in a high arctic environment. *Arctic and Alpine Research* 12(1): 1–10.

Campbell, D.R. 1987. Interpopulational variation in fruit production: the role of pollination-limitation in the Olympic Mountains. *American Journal of Botany* 74: 269–273.

Charnov, E.L. 1976. Optimal foraging, the marginal value theorem. *Theoretical Population Biology* 9: 129–136.

Cruden, R.W. 2000. Pollen grains: why so many? *Plant Systematics and Evolution* 222: 143–165

Darwin, C. 1859. *On the Origins of Species by Means of Natural Selection*, London: John Murray, 247.

547 Darwin, C. 1876. *The effects of cross and self fertilisation in the vegetable kingdom*,
548 London: John Murray, 147.
549 Dunn, D.B. 1956. The breeding systems of Lupinus, group Micranthi. *The American*
550 *Midland Naturalist* 55: 443–472.
551 Etcheverry, A., Aleman, V., Figueroa-Fleming, M.M., López-Spahr, T., Gomez, D.,
552 Yanez, C.A., Figueroa-Castro, D.M., and Ortega-Baes, P. 2012. Pollen: ovule ratio and
553 its relationship with other floral traits in Papilionoideae (Leguminosae): an evaluation
554 with Argentine species. *Plant Biology* 14: 171–178
555 Gori, D.F. 1989. Floral color change in *Lupinus argenteus* (Fabaceae): why should
556 plants advertise the location of unrewarding flowers to pollinators? *Evolution* 43: 870–
557 881.
558 Goergen, E., Chambers, J. C., and Blank, R. 2009. Effects of water and nitrogen
559 availability on nitrogen contribution by the legume, *Lupinus argenteus* Pursh. *Applied*
560 *Soil Ecology* 42(3): 200–208.
561 Harder, L.D. 1990. Pollen removal by bumble bees and its implications for pollen
562 dispersal. *Ecology* 71(3): 1110–1125.
563 Harder, L.D., and Barrett, S.C. 1992. The energy cost of bee pollination for *Pontederia*
564 *cordata* (Pontederiaceae). *Functional Ecology* 6: 226–233.
565 Harder, L.D., and Barrett, S.C. 1995. Mating cost of large floral displays in
566 hermaphrodite plants. *Nature* 373: 512–515.
567 Harder, L.D., and Thomson, J.D. 1989. Evolutionary options for maximizing pollen
568 dispersal of animal-pollinated plants. *American Naturalist* 133: 323–344.

569 de Jong, T.J., Waser, N.M., and Klinkhamer, P.G. 1993. Geitonogamy: the neglected
570 side of selfing. *Trends in Ecology and Evolution* 8: 321–325.
571 Heinrich, B. 1976. The foraging specializations of individual bumblebees. *Ecological*
572 *Monographs* 46(2): 105-128.
573 Irwin, R. E., and Maloof, J. E. 2002. Variation in nectar robbing over time, space, and
574 species. *Oecologia* 133(4): 525-533.

575 Kearns, C.A., and Inouye, D.W. 1993. *Techniques for pollination biologists*, Niwot:
576 University Press of Colorado, 155 and 289.
577 King, C., Ballantyne, G., and Willmer, P.G. 2013. Why flower visitation is a poor proxy
578 for pollination: measuring single-visit pollen deposition, with implications for pollination
579 networks and conservation. *Methods in Ecology and Evolution* 4: 811–818.
580 Klinkhamer, P.G., and de Jong, T.J. 1993. Attractiveness to pollinators: a plant's
581 dilemma. *Oikos* 66: 180–184.
582 Klinkhamer, P.G.L., de Jong, T.J.D., and Metz, J.A.J. 1994. Why plants can be too
583 attractive -- a discussion of measures to estimate male fitness. *Journal of Ecology* 82:
584 191–194.
585 Kudo, G. 2003. Anther arrangement influences pollen deposition and removal in
586 hermaphrodite flowers. *Functional Ecology* 17: 349–355.
587 Kuznetsova, A., Brockhoff, P.B., and Christensen, R.H.B. 2015. Package lmerTest. R
588 package version 2. R Foundation for Statistical Computing, Vienna, Austria. Website:
589 https ://cran.r-proje ct.org/web/packa ges/lmerT est/index.html.
590 Lefcheck, J. S. 2016. piecewiseSEM: Piecewise structural equation modelling in r for
591 ecology, evolution, and systematics. *Methods in Ecology and Evolution* 7(5), 573-579.

592 Luo, E.Y., Ogilvie, J.E., and Thomson, J.D. 2014. Stimulation of flower nectar
593 replenishment by removal: a survey of eleven animal-pollinated plant species. *Journal*
594 *of Pollination Ecology* 12: 52–62.
595 Macior, L.W., Ya, T., and Zhang, J. 2001. Reproductive biology of *Pedicularis*
596 (Scrophulariaceae) in the Sichuan Himalaya. *Plant Species Biology* 16: 83–89.
597 Morris, W. F., and Doak, D. F. 1998. Life history of the long-lived gynodioecious cushion
598 plant *Silene acaulis* (Caryophyllaceae), inferred from size-based population projection
599 matrices. *American Journal of Botany*, 85(6): 784–793.

600 Müller, H. 1873. *Die Befruchtung der Blumen durch Insekten und die gegenseitigen*
601 *Anpassungen beider: ein Beitrag zur Erkenntniss des ursächlichen Zusammenhanges*
602 *in der organischen Natur*, Leipzig: Engelmann, 243.

603 Muchhala, N., Brown, Z., Armbruster, W. S., and Potts, M. D. 2010. Competition drives
604 specialization in pollination systems through costs to male fitness. *The American*
605 *Naturalist* 176(6): 732–743.

606 Ogilvie, J. E., and Forrest, J. R. 2017. Interactions between bee foraging and floral
607 resource phenology shape bee populations and communities. *Current Opinion in Insect*
608 *Science* 21: 75-82.

609 Ordano, M., and Ornelas, J.F. 2005. The cost of nectar replenishment in two epiphytic
610 bromeliads. *Journal of Tropical Ecology* 21: 541–547.
611 Ornelas, J. F., Ordano, M., De-Nova, A. J., Quintero, M. E., & Garland Jr, T. 2007.
612 Phylogenetic analysis of interspecific variation in nectar of hummingbird-visited plants.
613 *Journal of Evolutionary Biology* 20(5): 1904-1917

614 Parachnowitsch, A.L., Manson, J.S., and Sletvold, N. 2018. Evolutionary ecology of
615 nectar. *Annals of Botany* 123: 247–261.
616 Pleasants, J. M. 1981. Bumblebee response to variation in nectar availability. *Ecology*
617 62(6): 1648-1661.
618 Price, M.V., Waser, N.M., Irwin, R.E., Campbell, D.R., and Brody, A.K. 2005. Temporal
619 and spatial variation in pollination of a montane herb: a seven-year study. *Ecology* 86:
620 2106–2116.
621 Pyke, G. H. 1978. Optimal foraging in hummingbirds: testing the marginal value
622 theorem. *American zoologist*, 18(4): 739-752.
623 Pyke, G.H. 1991. What does it cost a plant to produce floral nectar? *Nature* 350: 58–59.
624 Queller, D. 1997. Pollen removal, paternity, and the male function of flowers. *American*
625 *Naturalist* 149: 585–594.
626 Rabie, A. L., Wells, J. D., and Dent, L. K. 1983. The nitrogen content of pollen protein.
627 *Journal of apicultural research* 22(2): 119–123.
628 Simpson, B.B., Neff, J.L. 1983. Evolution and diversity of floral rewards. In: Jones CE,
629 Little RJ eds. *Handbook of Experimental Pollination Ecology*. New York: Van Nostrand
630 Reinhold, 277–293.
631 Stanton, M.L., Snow, A.A., and Handel, S.N. 1986. Floral evolution: attractiveness to
632 pollinators increases male fitness. *Science* 232: 1625–1627.
633 Stpiczyńska, M. 1995. The structure of floral nectaries of some species of *Vicia* L.
634 (Papilionaceae). *Acta Societatis Botanicorum Poloniae*, 64(4): 327–334.
635 Thomson, J.D. 1986. Pollen transport and deposition by bumble bees in *Erythronium*:
636 influences of floral nectar and bee grooming. *The Journal of Ecology* 74: 329–341.

637 Thomson JD. 1981. Field measures of flower constancy in bumblebees. *American*
638 *Midland Naturalist* 105(2):377–380.
639 Thomson, J.D., Wilson, P., Valenzuela, M., and Malzone, M. 2000. Pollen presentation
640 and pollination syndromes, with special reference to Penstemon. *Plant Species Biology*
641 15: 11–29.
642 Thøstesen, A.M., and Olesen, J.M. 1996. Pollen removal and deposition by specialist
643 and generalist bumblebees in *Aconitum septentrionale*. *Oikos* 77: 77–84.
644 Tong, Z.Y., Wang, X.P., Wu, L.Y., and Huang, S.Q. 2018. Nectar supplementation
645 changes pollinator behaviour and pollination mode in *Pedicularis dichotoma*:
646 implications for evolutionary transitions. *Annals of Botany* 123: 373-380.
647 Venables, W.N., and Ripley, B.D. 2002. *Modern applied statistics with S*, New York:
648 Springer-Verlag, 183–206.
649 Wainwright, C.M. 1978. The floral biology and pollination ecology of two desert lupines.
650 *Bulletin of the Torrey Botanical Club* 105: 24–38.
651 Waser, N. M., and Price, M. V. 2016. Drought, pollen and nectar availability, and
652 pollination success. *Ecology* 97(6): 1400-1409.
653 Waser, N.M., and Williams, C.F. 2001. Inbreeding and outbreeding. In: Fox C.W., Roff
654 D.A., and Fairbairn, D.J., eds. *Evolutionary Ecology, Concepts and Case Studies*.
655 Oxford: Oxford University Press, 84–96.
656 Wilson, P., Thomson, J.D., Stanton, M.L., and Rigney, L.P. 1994. Beyond floral
657 Batemania: gender biases in selection for pollination success. *American Naturalist* 143:
658 283–296.

FIGURE LEGENDS

FIGURE 1 *Lupinus argenteus* corolla structure and the artificial nectar placement method and location. (A) Inflorescence with micro-pipette tip placed between keel (1) and wing (2) petals to deposit artificial nectar. Banner petal (3) is also labeled. (B) One wing petal removed to reveal keel tip (4), body of fused keel petals (5), and 2 µL droplet of artificial nectar (6).

FIGURE 2 A typical pollen collecting visit by a bumble bee (*Bombus bifarius* worker pictured) to an unmanipulated (not treated with artificial nectar) *Lupinus argenteus* inflorescence. (A) *Bombus bifarius* bearing corbicular pollen loads approaching a *Lupinus argenteus* flower with glossa extended (indicated by arrow); (B) a bee braces her head against banner petal and grips wing petals with her forelegs. As head is pressed into banner, glossa extends into gap between wing and keel petals (indicated by arrow); (C) Wing petals are depressed away from keel with fore and midlegs, causing expulsion of a pollen droplet from keel pore onto anterior abdomen segments. Glossa is no longer in the keel-wing gap. The keel surface within the keel-wing gap (indicated by arrow) is not in contact with the body of the bee at any point.

FIGURE 3 Absolute volumes (A) and differences in volume (B) of artificial nectar recovered from paired visited and virgin flowers within single stalks of *Lupinus argenteus* following nectar removal by bees. Visited flowers contained less artificial nectar than unvisited flowers on average. Bars are boxplots, with lower and upper ends of boxes depicting the lower and upper quartiles, respectively. Solid bands indicate medians. Whiskers extend across the data range. N = 9 plants.

FIGURE 4 Violin plot of mean visit duration to control (N = 1,367 flowers probed, 73 plant visits) and nectar added flowers (N = 1,580 flowers probed, 98 flower visits). Boxes are upper and lower quartiles; points are medians; whiskers extend across the data range; curves are rotated probability density functions.

FIGURE 5 Single-visit pollen deposition increased with flower visit duration (sec). Points are raw data, with treatments differentiated by symbol. Band represents 95% CI.

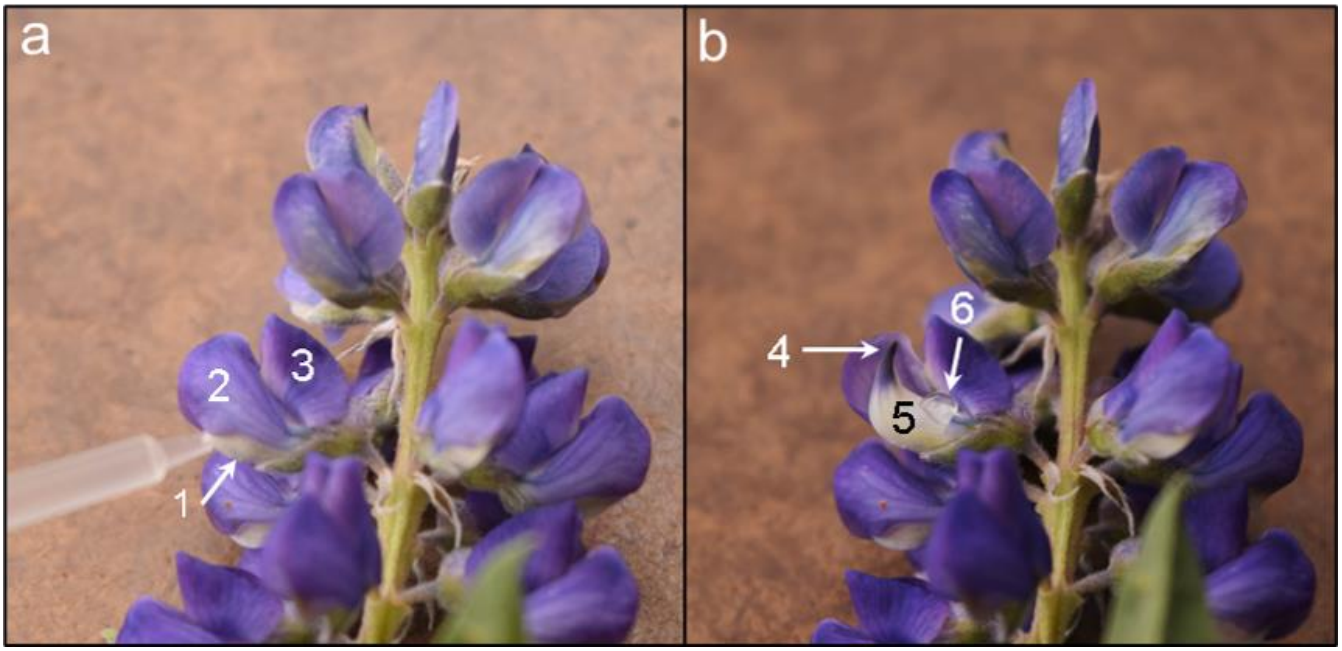
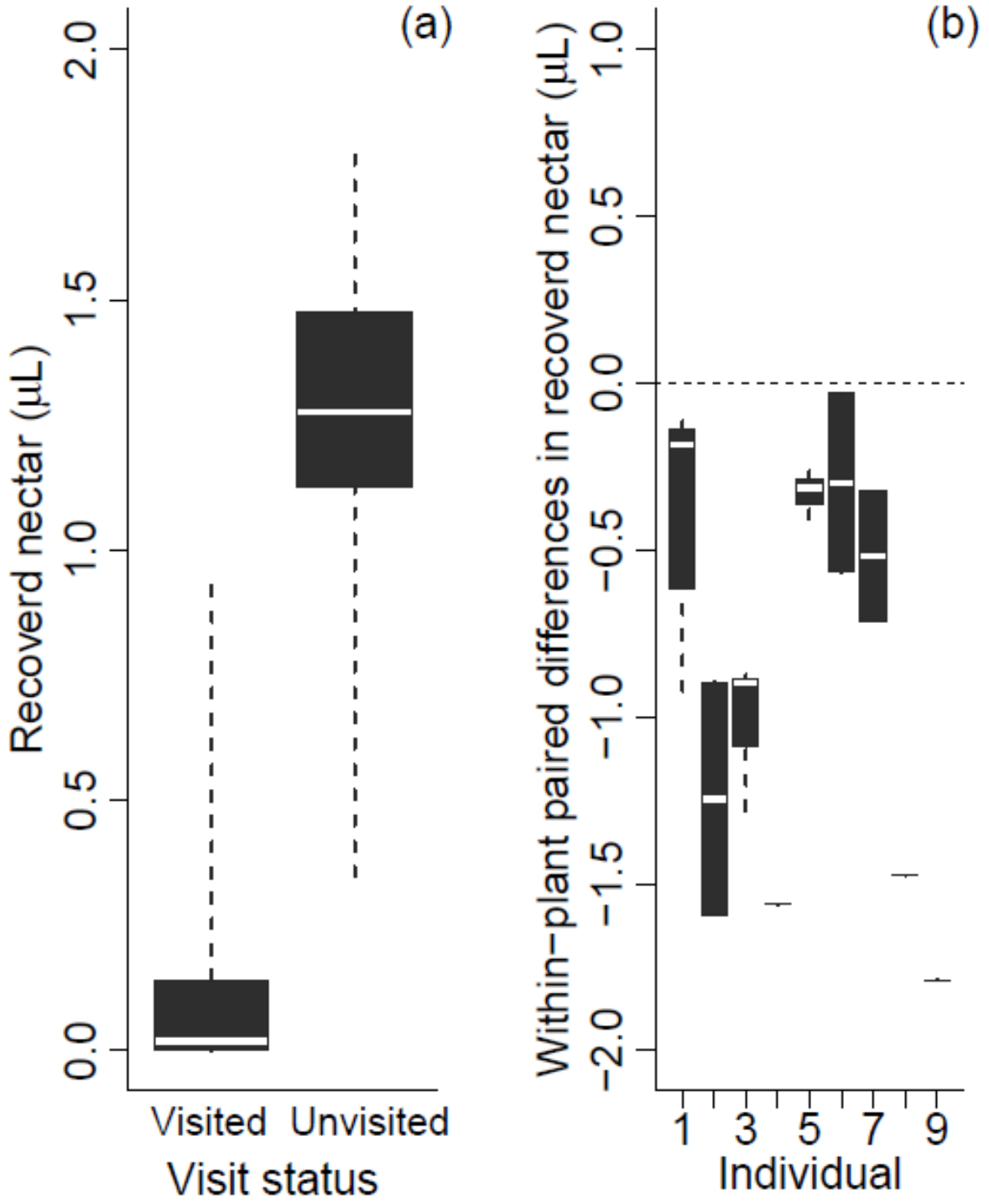


Figure 1.

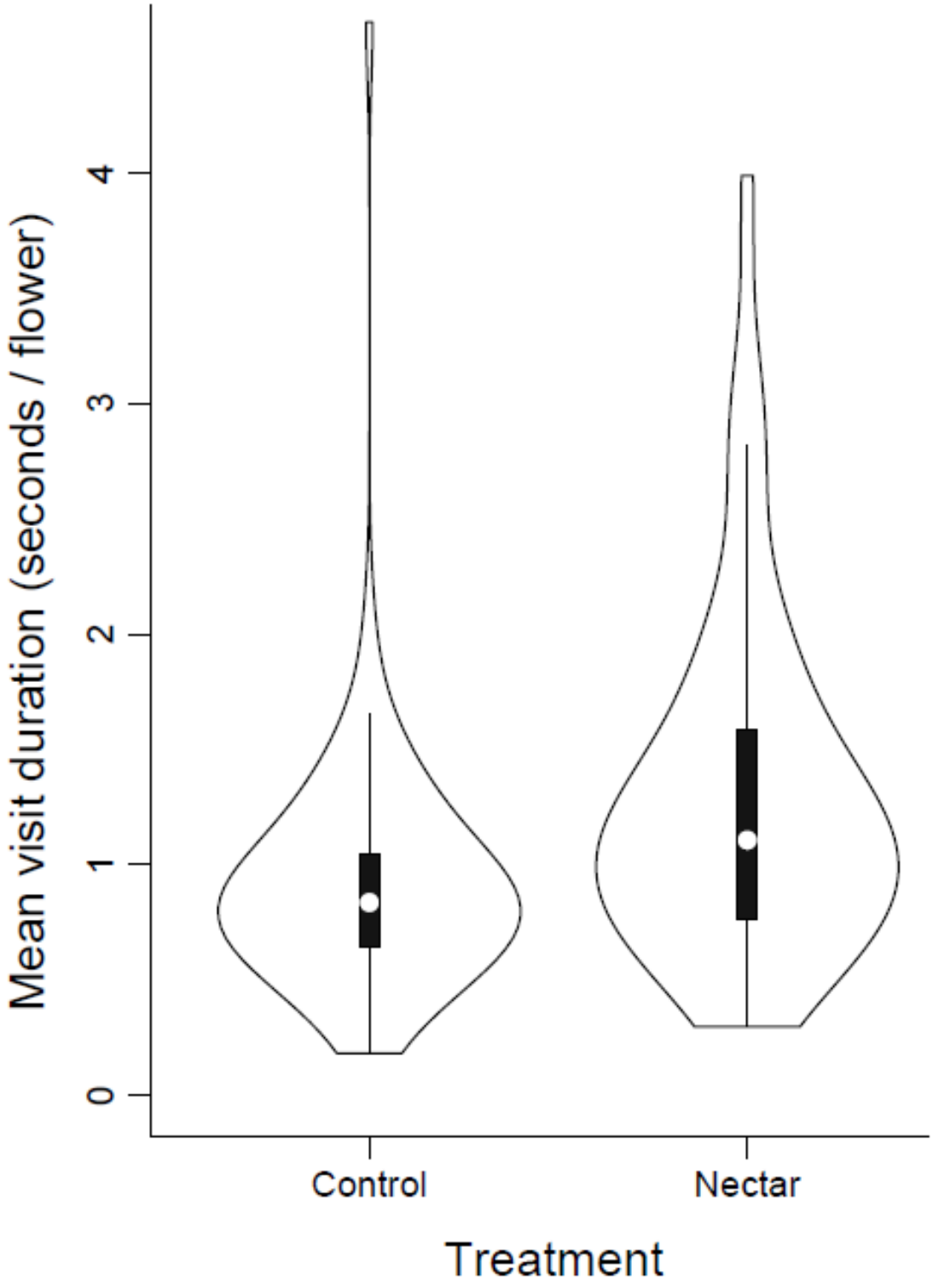
691 

692 Figure 2.



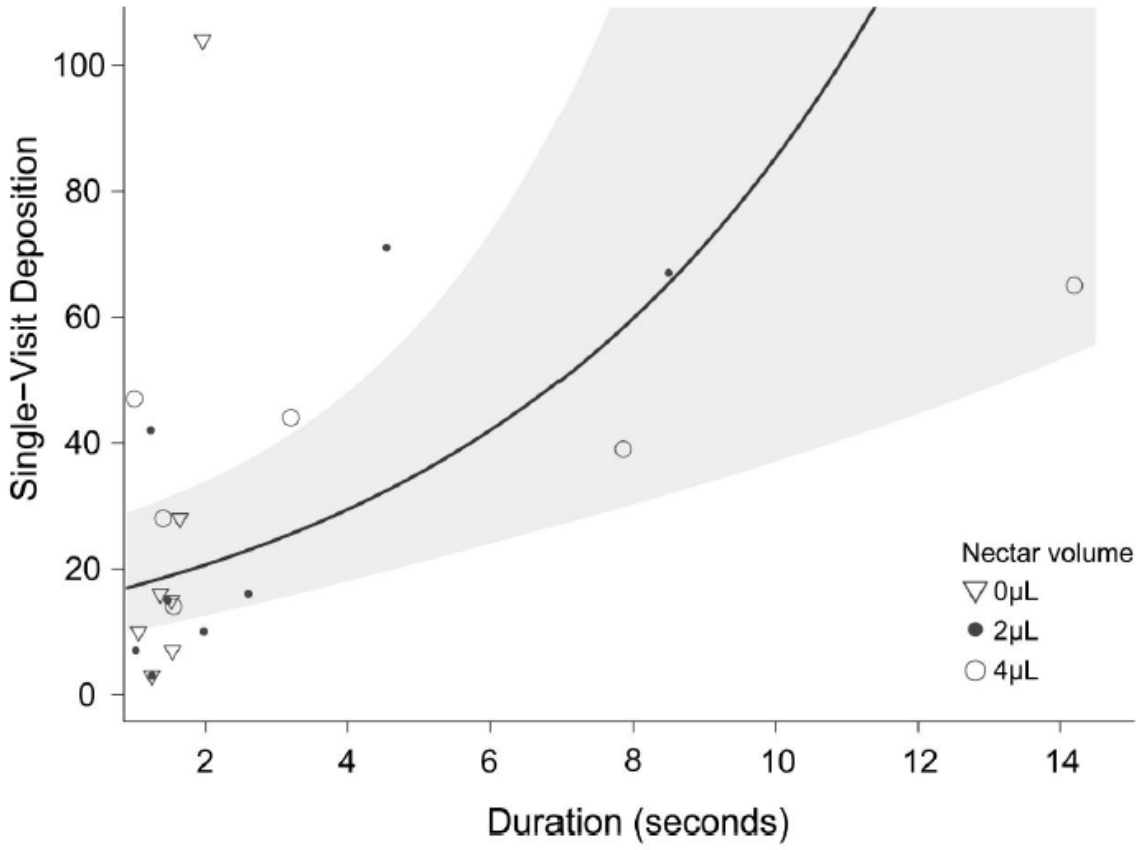
693

694 Figure 3.



695

696 Figure 4.



697

698 Figure 5.

699