

ABSTRACT

HEILING, JACOB MICHAEL. Floral Reward Strategies, Visitor Behavior, and Plant Reproductive Outcomes. (Under the direction of Rebecca Irwin).

Floral rewards are produced by angiosperms in part or exclusively to entice pollinators. The many types of floral rewards that plants produce, including nectar and pollen, differ in their costs and benefits, constituting alternative “reward strategies”. The reward strategy a plant employs shapes its interactions with floral visitors – mutualists, antagonists, and commensals. In turn, the fitness outcomes of these interactions define the ecological and evolutionary trajectories of rewards and other floral traits. In this thesis, I describe four studies that address aspects of two central questions in the study of floral rewards: 1) How do rewards, and the behavior of floral visitors in response to those rewards, affect plant reproduction? And 2) How do plants balance the need to provide rewards to pollinators and the need to protect and disseminate gametes?

Nectar robbing, the removal of nectar through holes pierced in flowers, is common in plants with hidden floral nectaries. Nectar robbing can reduce plant reproduction but, surprisingly, sometimes fails to do so. Using observations and experiments in *Corydalis caseana*, we found evidence for three mechanisms explaining neutral nectar robbing outcomes. First, pollinators failed to discriminate against less rewarding experimentally robbed flowers. Second, *C. caseana* flowers were more often visited by pollinators than nectar robbers. Third, pollen loads exceeded levels required for stigma saturation. These mechanisms buffer the effects of nectar robbing on plant reproduction, allowing nectar robbing to function as a commensalism.

Pollen is also used as a floral reward. Yet, pollen consumed by pollinators represents lost reproductive opportunities for plants. To better understand why many species are pollen-rewarding, we added artificial nectar to strictly pollen-rewarding *Lupinus argenteus*, simulating a novel nectar + pollen rewarding phenotype, and compared pollinator behavior and plant

reproduction between nectar-added and control plants. Pollinators collected pollen and nectar from nectar-added flowers and spent longer on nectar-added flowers than controls. Pollen deposition increased with visit duration. However, we found no effect of nectar-addition on female reproduction. In pollen-rewarding plants, providing an additional reward without also limiting pollinators' access to pollen is unlikely to provide any further benefit.

Next, we considered how the pollen-reward strategy affects male mating success. We modeled the effects of pollen production and pollen package size on pollen donation, accounting for pollen loss and pollinator reward size preference. We found that increasing pollen production and dividing pollen among many large packages may help pollen-rewarding plants maximize male mating success. The conflicting constraints of pollinator preference and grooming on pollen donation may explain the large pollen investments observed in many pollen-rewarding plants, despite the cost of lost mating opportunities.

Finally, given the potential for negative effects of pollen consumption, we explored pollen defensive chemistry. We investigated whether pollen secondary chemistry in three pollen-rewarding *Lupinus* species (*L. argenteus*, *L. bakeri*, and *L. sulphureus*) better reflected the need to defend pollen from consumption or to reward pollinators. Using gas-chromatography, we characterized the alkaloid profiles in four tissues (pollen, flowers, leaves, and stems) and compared profiles among tissues within individuals. In *L. argenteus* and *L. sulphureus*, alkaloid concentrations were lowest in pollen. We detected no alkaloids in *L. bakeri* pollen. Our results indicate that pollen secondary chemistry may better reflect the need to reward pollinators than the need to defend pollen in these species.

Floral rewards are the foundation of plant—pollinator mutualisms. This work contributes to a growing effort to interpret the significance and consequences of floral reward strategies in the contexts of plant reproductive ecology, species interactions, and the evolution of floral traits.

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Floral Reward Strategies, Visitor Behavior, and Plant Reproductive Outcomes.

by
Jacob Michael Heiling

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APPROVED BY:

Rebecca E. Irwin
Committee Chair

Judith L. Bronstein

Nicholas M. Haddad

Kevin Gross

William F. Morris

DEDICATION

To Karen Susan Maresh Heiling, who taught me to love and respect the natural world.

And to Robert Michael Heiling, who taught me the beauty of inquiry.

BIOGRAPHY

A third-generation Czech—German Texan, I was born in San Angelo, TX and grew-up on the Texas Gulf Coast and in northern Georgia. I have always been fascinated by living things and wild places, and my family has encouraged these interests for as long as I can remember. Some of my earliest memories are of my mother teaching me the names of plants (and telling me which ones were edible) and spending evenings on the porch with my father, speculating about space, time, and evolution. I graduated from Memorial High School in Victoria, TX in 2004, after which I worked full-time. In January 2006 I began work at Kerry Alternative Technology, an off-the-grid farm in Co. Kerry, Republic of Ireland. In 2007 I enrolled part-time at Victoria Community College while working full-time. In 2009 I moved to Austin Texas, intent on pursuing a B.S. in Biology at the University of Texas at Austin. There, I met Gina Calabrese, who has been my partner in life and science ever since, and I met Jay Falk, who became a close friend and colleague and with whom I subsequently worked several of my first field jobs. Jay introduced me to Dan Bolnick's lab, where I assisted Christine Parent in *Tribolium* evolution research, and eventually ran an experiment of my own. Christine and Dan were very generous with their time and were excellent mentors. As a student at UT, I was fortunate enough to work for Larry Gilbert (maintaining greenhouses, among other odd-jobs) and Tom Juenger. I met David Lowry, who was my REU advisor in 2012, and who became one of my most influential mentors, an incredible advocate, and a good friend. I graduated from the University of Texas with a B.S. in Biology in 2013, after which I worked as a post-bac lab tech in Lila Fishman's lab in Missoula, MT. I joined the Irwin lab as a Ph.D. student in 2014, at Dartmouth College. I moved with the Irwin lab to NC State in 2015.

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The work presented here took place on Abenaki, Ute, and Cherokee traditional territories

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To Mishka, my best friend – words are not enough.

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For to the bee a flower is a fountain of life, and to the flower a bee is a messenger of love

– Kahlil Gibran, *The Prophet*, 1923

CHAPTER 1: Why are some plant—nectar robber interactions commensalisms?

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ABSTRACT

Many plants that bear hidden or recessed floral nectar experience nectar robbing, the removal of nectar by a floral visitor through holes pierced in the corolla. Although robbing can reduce plant reproductive success, many studies fail to find such effects. We outline three mechanistic hypotheses that can explain when interactions between plants and nectar-robbers should be commensal rather than antagonistic: the non-discrimination (pollinators do not avoid robbed flowers), visitor prevalence (robber visitation is rare relative to pollinator visitation), and pollen saturation (stigmas receive sufficient pollen to fertilize all ovules with one or very few pollinator visits) hypotheses. We then explore these mechanisms in the North American subalpine, bumble bee-pollinated and nectar-robbed plant *Corydalis caseana* (Fumariaceae). We first confirmed that the effects of nectar robbing on female reproductive success were neutral in *C. caseana*. We then tested the three mechanisms underlying these neutral effects using a combination of observational studies and experiments. We found evidence for all three mechanisms. First, consistent with the non-discrimination hypothesis, pollinators failed to discriminate against experimentally robbed flowers or inflorescences even though naturally robbed flowers offered significantly lower nectar rewards than unrobbed flowers. Second, *C. caseana* was more commonly visited by pollinators than by nectar robbers, in accordance with the visitor prevalence hypothesis. Third, stigmas of unvisited flowers as well as those visited once by pollinators were saturated with pollen, with all stigmas bearing pollen loads several

orders of magnitude higher than the number of ovules per fruit, consistent with the pollen saturation hypothesis. Our investigation of the mechanisms driving the commensal outcome of nectar robbing in this system deepens our understanding of the ecology of nectar robbing and contributes to a more general understanding of the variation in the outcomes of interactions between species.

INTRODUCTION

Mutualisms are prone to exploitation when individuals collect a reward or obtain a service from their partner without reciprocating. The fitness effects imposed by exploiters have traditionally been assumed to be negative (Jones et al., 2015). For example, in an early description of the exploitation of nectar rewards in flowers by non-pollinating visitors, Darwin supposed that “...all plants must suffer in some degree when bees obtain their nectar in a felonious manner...” (Darwin, 1876). Indeed, exploitation can be detrimental to host fitness because the resources invested in the reward or service are lost, potentially making future interactions with mutualist partners less likely (Irwin and Brody, 1998), and/or increasing the metabolic cost of reward production for species that replenish rewards (e.g., nectar) upon removal (Pyke et al., 1988). However, the outcomes of species interactions depend on the interplay between traits and processes unique to the particular web of interaction partners and the larger community in which they are imbedded and may not coincide with the predictions we might make based on first principles. There is growing recognition that exploiters of mutualisms do not always, or even typically, impose negative fitness effects on their hosts (Jones et al., 2015). In these cases, the exploiter may be acting as a commensal: that is, it may profit from its partner, while the partner experiences neither positive or negative fitness consequences from the interaction. For example, some non-pollinating fig wasps consume sterile fig tissue and exact no reproductive cost on their plant hosts (Bronstein, 1991). While a great deal of research has explored the causes and consequences of negative species interactions (predation, competition, parasitism) (Gurevitch et al., 2000) and, to growing extent, positive species interactions (mutualism and facilitation) (Bronstein, 2015), relatively little work has explored commensalism, despite its ubiquity in nature (e.g., Palumbi, 1985; Sáyago et al., 2013).

One of the best-studied examples of exploitation of mutualisms is the interaction between animal-pollinated plants and nectar robbers (reviewed in Irwin et al., 2010). Nectar robbers are floral visitors that feed on nectar via holes made in flowers, often without transferring pollen. The effect of nectar robbers on plant reproduction ranges from negative to neutral to positive (Olesen, 1996; Maloof and Inouye, 2000; Irwin et al., 2001). Plants can experience direct negative effects of robbing through damage to floral reproductive tissues (McDade and Kinsman, 1980), and indirect negative effects through decreased attractiveness of robbed flowers to pollinators (Irwin and Brody, 1999). Conversely, when visitation by pollinators does not limit seed production, decreased attractiveness due to nectar removal by robbers may actually benefit plant reproduction by increasing pollinator flight distances between plants, possibly reducing rates of self-pollination and bi-parental inbreeding (Zimmerman and Cook, 1985; Maloof, 2001). Robbers may also positively affect plant reproduction if they pollinate flowers while robbing (Olesen, 1996; Maloof and Inouye, 2000). Many studies document both negative and positive effects of robbing, and several have explored the mechanisms underlying these effects (Inouye, 1983; Burkle et al., 2007). However, while roughly one third of the studies of nectar robbing find no effect of robbing on plant fitness (Maloof and Inouye, 2000) and some of these offer plausible post-hoc explanations for it (Inouye, 1983; Hazlehurst and Karubian, 2016), few studies have directly explored mechanisms driving neutral effects (but see Morris, 1996). Here we explicitly formulate and test mechanistic hypotheses that might explain neutral effects of nectar robbing on plant reproduction.

We suggest that at least three, non-mutually exclusive hypotheses could explain neutral effects of nectar robbing. First, pollinators may not discriminate between robbed and unrobbed flowers (the *non-discrimination hypothesis*). Nectar-robbed flowers often have lower nectar

standing crops than unrobbed flowers (Richardson and Bronstein, 2012). While pollinators sometimes avoid robbed flowers due to low nectar volumes (e.g., Dohzono, et al., 2008; Zhang et al., 2009), such behavior is not ubiquitous (Fumero-Cabán and Meléndez-Ackerman, 2013) and may be dependent on the pollinator species (Irwin et al., 2001). This hypothesis predicts that robbing has a neutral effect on plant reproduction even in pollen-limited populations, because robbing does not change the behavior of pollinators. Second, the *visitor prevalence hypothesis* predicts that if the ratio of robber-to-pollinator visitation frequency is low enough, then robbing may have a neutral effect even if there is some pollinator discrimination against robbed flowers. This mechanism may be especially relevant for female mating success, which often peaks with fewer visits from pollinators than male mating success (Carlson, 2007). Third, the *pollen saturation hypothesis* predicts that even moderate to large reductions in pollinator visitation to nectar-robbed plants and flowers may not translate into differences in female reproductive success. This mechanism could operate when plant reproduction is not pollen-limited, either due to high pollen receipt relative to ovules per flower or due to autogamous selfing in self-compatible species with little inbreeding depression (Burkle et al., 2007). The *visitor prevalence* and *pollen saturation* hypotheses predict the same outcome (visitation by pollinators is high enough to avoid pollen limitation). However, they are distinct from one another because the mechanisms driving that outcome differ. The *visitor prevalence hypothesis* relies on a community-based mechanism, while the *pollen saturation hypothesis* relies primarily on floral design and plant mating system. While these three mechanisms have, to some extent, been suggested previously and partially explored, no studies have formalized a discrete set of hypotheses regarding mechanisms driving neutral effects of robbing or to test such a set of hypotheses together in a single system.

The goal of this study was to test these three hypotheses empirically in a native North American plant, *Corydalis caseana* A. Gray (Fumariaceae), which is both robbed and pollinated by bumble bees. Prior research on this species documented neutral effects of nectar robbing on female components of plant reproduction (Maloof, 2000, 2001); thus, this system provided an opportunity to test the mechanisms driving these effects with respect to female reproductive success. Given the consistency of evidence for spatio-temporal variation in the outcome of species interactions (Chamberlain et al., 2014), we first conducted observations and experiments to verify that the effects of robbing on female components of *C. caseana* reproduction were neutral in the populations and years in which we worked. We then used a series of observations and experiments to explore the mechanisms driving these effects. We first assessed whether there was a reward-based motivation for pollinators to discriminate against robbed plants and flowers, by testing the assumption that nectar standing crops differ between robbed and unrobbed flowers. We confirmed that robbed flowers had significantly lower nectar standing crops compared to unrobbed flowers. We then tested the non-discrimination hypothesis through a field experiment designed to answer the question: Do pollinators discriminate against robbed plants and flowers? If pollinators visit robbed and unrobbed flowers at similar rates, as the non-discrimination hypothesis predicts, then robbing status should not influence pollen receipt and seed set, barring any physical damage to the gynoecium or androecium during robbing. To test the visitor prevalence hypothesis, we asked: How common are pollinator visits relative to nectar robber visits? Plants may escape the negative effects of robbing if robber visits are rare relative to those from pollinators. Finally, we tested the pollen saturation hypothesis by asking: How do single visits from pollinators affect stigmatic pollen loads? High single-visit pollen receipt, as well as high levels of autogamous pollen transfer, in self-compatible species could negate any possible

plant-reproduction effects of pollinator discrimination between robbed vs. unrobbed flowers. Our results demonstrate how nectar robbing can produce a commensal outcome, using general hypotheses that may be relevant to all plant-pollinator-robber interaction webs.

METHODS

Study System

Our field sites were in the Elk Mountains of Gunnison County, Colorado, USA near the Rocky Mountain Biological Laboratory (RMBL). We worked in three sites: Kebler Pass (38.86558° N, -107.10528° W; 3086.40 masl), Poverty Gulch (38.95615° N, -107.07713° W; 2917.55 masl), and Washington Gulch (38.95997° N, -107.03379° W; 3149.80 masl). All sites were in wet subalpine meadows with similar floral communities and bee assemblages. *Corydalis caseana* A. Gray (Fumariaceae) is an herbaceous hermaphroditic perennial occurring in wet meadows, seeps, and drainages of the subalpine zone of North America's mountain west. It grows in dense stands with stalks reaching 1-1.5 m in height, and mature plants can bear approximately 20 stalks (Maloof, 2000). Stalks bear numerous terminal racemose, occasionally branching, inflorescences with approximately 5-70 flowers each (Maloof, 2000). The bilaterally symmetrical flowers bear a single nectar spur. The flowers are hermaphroditic and do not exhibit dichogamy or herkogamy (Maloof, 2000). While pollen is readily transferred to the stigmas autogamously, flowers are only partially self-compatible, with seed set approximately 1.5 times higher in outcrossed flowers; open pollinated flowers produce a mean of 5 ± 0.8 seeds/fruit (± 2 SE) (Maloof, 2000). Flowers produce nectar (35% sugar) at a rate of approximately 2 μ L/day (Maloof, 2000). In the West Elk Mountains, bumble bees (*Bombus* spp.) are the most common floral visitors to *C. caseana*. *Bombus appositus* is the most common pollinator, responsible for

>50% of all visits to the species in some years (Maloof, 2000). Less frequent, more spatially and temporally variable visitors include *Bombus flavifrons*, *B. bifarius*, *B. balteatus*, *B. mixtus*, *B. frigidus*, *B. rufocinctus*, *B. nevadensis*, and *B. fervidus*, as well as some non-*Bombus* bees, hummingbirds, and butterflies.

Corydalis caseana is also commonly nectar-robbed by at least two bumble bee species, *Bombus occidentalis* and *B. mixtus*. *Bombus occidentalis* is historically the most common nectar robber on *C. caseana* and was responsible for approximately 30% of all floral visits observed in some sites and years (Maloof, 2000). *Bombus occidentalis* and *B. mixtus* can act both as primary nectar robbers (making robbing holes in flowers and removing nectar) and as secondary nectar robbers (removing nectar from holes already present) on *C. caseana*. These robbers do not damage reproductive or nectar-producing structures while primary or secondary robbing; thus, robbers do not directly interfere with fertilization or seed development in this species. *Corydalis caseana* is consistently and heavily robbed, with > 90% of flowers bearing robbing holes in many years and sites and experiences high levels of secondary robbing (Irwin and Maloof, 2002). Despite heavy nectar robbing, a previous study found neutral effects of robbing on *C. caseana* fruit and seed set (Maloof, 2001). The mechanisms driving these neutral effects are unknown, and we investigated them here.

Field Methods

Are the effects of nectar robbing on female function neutral?

To confirm that the effects of nectar robbing on female reproduction were neutral in the populations in our study, consistent with Maloof (2000) from field work conducted in 1996, we

used observations and experiments in 2014 and 2015 to compare pollen receipt, pollen-tube growth, fruit set, and seed set between robbed and unrobbed plants and flowers.

I. Observational study

The number of pollen tubes per stigma is a proxy for female mating success, as the number of pollen tubes in a stigma sets an upper limit on the number of ovules that can be fertilized in an ovary. We compared pollen tube growth in unrobbed and naturally robbed *C. caseana* flowers collected near peak bloom (population-level) from three sites in 2014 (Kebler Pass on 14 July, Poverty Gulch on 10 and 15 July, and Washington Gulch on 11 July). We sampled up to 10 plants per site and collected up to five robbed (bearing at least one robbing hole) and five unrobbed flowers of similar age from each plant. We collected 311 styles from 32 plants, and successfully processed 249 styles from 29 plants.

In the field, we fixed each flower in 3:1 (volume : volume) 95% ethanol : glacial acetic acid to arrest pollen-tube growth. In the lab, we rinsed pistils in DI water and transferred them to new vials containing 4:1 (volume : volume) 1% basic fuchsin : 1% fast green to stain pollen tubes for visualization *via* white light transmission. After 24 h, we rinsed pistils in DI water and transferred them to vials with 88% lactic acid to clear and soften them. After 5 d in lactic acid, we mounted the styles on microscope slides using clear fingernail varnish (Kearns and Inouye, 1993). Using a compound microscope, we counted the number of pollen tubes at the receptacle end of each style at 1000x magnification. For each plant, we calculated the mean number of pollen tubes in robbed vs. unrobbed flowers.

Statistical analysis: We performed all statistical analyses (here and below) in R version 3.4.0 (R Core Team, 2017). We compared mean number of pollen tubes between robbed and unrobbed flowers using a mixed effects model, fit by maximum likelihood (ML), with robbing

status (robbed vs. unrobbed) as a fixed effect and site and plant as random effects, with plant nested within site. To assess the effect of robbing status on pollen tube count, we performed a likelihood ratio test by comparing the full model to a model with the same random effects, but with intercept as the only fixed effect. We constructed the generalized linear mixed effects models (GLMM) in *lme4* (Bates, 2010) in R (here and below) and used *lmerTest* (Kuznetsova et al., 2015) to perform inference tests for GLMMs.

II. Experimental study

We experimentally manipulated the frequency of nectar-robbed flowers at one site (Kebler Pass) and measured subsequent effects on pollen receipt, fruit set, and seed set from 2-24 July 2015. We haphazardly assigned plants to one of three treatments with 5 plants per treatment: no robbing (*control*), flowers robbed one time (*primary robbing*—*PR*), and flowers robbed daily (*continuous robbing*—*CR*). Because of the large number of flowers that *C. caseana* individuals bear (Maloof, 2001), it was not possible to treat every flower on a plant. Instead, we treated a subset (10-20%) of the flowers within each of five lateral racemes on each plant. We tracked treated flowers by placing a small mark on their corollas using a permanent marker. Marking corollas in this way does not affect visitation by pollinators or robbers (Maloof, 2000).

To apply robbing treatments, we made a small hole in the corolla with dissecting scissors and removed nectar with 5 μ L microcapillary tubes (Drummond Scientific, Broomall, Pennsylvania, USA). This method of artificial robbing mimics natural robbing by bumble bees (Burkle et al., 2007; Irwin and Brody, 1998; Richman et al., 2017). We fit clear plastic collars over the nectar spur of each flower to prevent any robbing for the control (unrobbed) treatment and secondary robbing for the PR treatment. We cut small windows in the collars we used for the

CR treatment to control for the effects of the collars on the other treatments while allowing subsequent experimental robbing with a microcapillary tube each day until flowers senesced. Collaring flowers in this way does not deter pollinators (Irwin and Brody, 1999; Morris, 1996). By using both the PR and CR treatments, we were able to test whether daily robbing in the CR treatment resulted in any additional plant fitness effects beyond the initial robbing event in the PR treatment (akin to Richman et al., 2017).

To measure pollen receipt, we collected the stigmas from focal flowers upon corolla senescence. At this point, pollen should have grown down the style and fertilized the ovules, so stigma collection should not have affected fruit or seed set. We mounted fresh stigmas on microscope slides using basic fuchsin gel (Kearns and Inouye, 1993). We imaged slides with a digital compound microscope and counted conspecific pollen using *Count Tool* in Adobe Photoshop Creative Cloud. We calculated average pollen receipt per flower per raceme.

To assess fruit and seed set, we monitored each flower until flower abscission, fruit abortion, or fruit set. We then collected mature fruits and counted the number of seeds per fruit. We estimated female function as 1) proportion fruit set per raceme (number of mature fruits/number of mature and aborted fruits) and 2) seed set per raceme (total number of seeds per raceme).

Statistical analyses: We tested whether robbing treatment affected mean conspecific pollen receipt per flower per raceme using a linear mixed effects model (ML) with robbing treatment (control, PR, CR) and the number of flowers per raceme as fixed effects. Because we sub-sampled racemes within plants, we included plant as a random effect in all models to prevent pseudoreplication. We square-root transformed mean pollen receipt to meet the assumption of Gaussian error distribution. We tested how robbing treatment affected proportion fruit set and

seed set per raceme using linear and generalized linear mixed effects models, respectively. We included robbing treatment as a fixed effect, number of flowers per raceme as a covariate in the proportion fruit set model, and number of mature fruits per raceme as a covariate in the seed set model. To test significance of fixed effects in all models, we ran likelihood ratio tests comparing the full models to null models with the same random effects but with intercept as the only fixed effect.

Non-discrimination hypothesis: Are nectar rewards diminished by robbing?

To assess the nectar reward availability in robbed and unrobbed flowers, we used 5 μL microcapillary tubes to measure the nectar standing crop of each flower that we collected for the pollen tube growth study ($N = 32$ plants across three sites). For each plant, we calculated the proportion of robbed and unrobbed flowers without nectar (treating nectar as either present or absent) and the mean nectar standing crop in robbed and unrobbed flowers.

Statistical analysis: We compared the proportion of robbed and unrobbed flowers that were empty of nectar using a linear mixed effect model (REML) with robbing status (robbed vs. unrobbed) as a fixed effect and plant as a random effect. We used a similar model with mean nectar standing crop in robbed and unrobbed flowers as the response. We dropped one individual from the analysis because a Grubbs Test (using the R package ‘*outliers*’; Komsta, 2011) revealed that the nectar standing crop value (4.17 μL) was an outlier ($G = 6.39$, $P < 0.0001$).

Non-discrimination hypothesis: Do pollinators discriminate between robbed and unrobbed flowers?

To test the prediction that plants escape negative effects of robbing because pollinators do not avoid robbed flowers, we measured pollinator visitation to experimentally robbed and control (unrobbed) flowers and stalks. At the Washington Gulch site on 29 and 30 June and 6 July 2016, we haphazardly assigned two treatments, robbed and unrobbed, to paired stalks on 77 plants. Each plant was only used for one day and was marked with flagging tape to prevent resampling on subsequent days. For flowers on stalks in the robbing treatment, we artificially robbed all flowers but did not place collars on flowers after robbing. We handled but did not cut holes in or remove nectar from, flowers on unrobbed stalks. We recorded the number of open flowers on each stalk (2 – 116 flowers/stalk, mean = 29.5 ± 1.5 SE). Once treatments were applied, we observed the patch for all floral visitors to marked stalks for 2 consecutive hours (between 10:00 and 16:00) on sunny to partly cloudy days, using multiple observers, resulting in >69.65 person-hours of observation. We used digital voice recorders to record the number of pollinator bouts received by each treatment stalk, the number of flowers visited per stalk per bout, the time spent per flower, and the identity of visitors. We identified pollinators to family for non-bumble bee pollinators and to species for bumble bees. We identified pollinators on the wing to the lowest taxonomic level we could achieve without netting them.

Statistical analyses: We tested how robbing treatment affected pollinator foraging behavior using GLMM (ML) for count data (total bouts received) and using liner mixed effects models (ML) for continuous data (mean time spent per flower and proportion of flowers visited per stalk). Count data were over-dispersed and zero-inflated; thus, we fit a zero-inflated GLMM with a negative binomial error distribution (Brooks et al., 2017). We constructed the generalized

linear mixed effects models (GLMM) using *glmmTMB* and used *lmerTest* to perform inference tests (Brooks et al., 2017). For all models, we performed backward model selection and compared AIC values to determine best fit models. Initial predictors included date, robbing treatment (robbed vs. unrobbed), and number of flowers open per stalk as fixed effects, and plant as a random effect (to account for pairing). For mean proportion of flowers probed and mean time spent per flower, we only used data from plants for which we observed visitation to both stalks (robbed and unrobbed), and we dropped one plant from the analysis because a Grubbs Test revealed that the mean time per flower was an outlier (14.5 and 4.85 s on the robbed and unrobbed flowers respectively), ($G = 6.69$, $P < 0.0001$). We natural-log transformed mean flowers visited and mean time spent per flower to meet the assumption of Gaussian error distributions.

Visitor prevalence hypothesis: How common are pollinator relative to nectar-robber visits?

To determine relative visitation frequencies by pollinators and robbers (both primary and secondary) at the plant population level, we recorded floral visitation to *C. caseana* on 16 days at three sites (five to six d per site) between 2 July and 9 August, 2014 for approximately two and a half h per site per d. The sampling period spanned roughly from just before the peak to the end of the flowering season of *C. caseana* at these sites. We used voice recorders to record each flower visit we observed to *C. caseana* and the type of visit, with each observer tracking only one kind of visitor (robbers or pollinators) during an observation session. We followed each visitor until it flew out of sight.

Statistical analyses

We summed visits of each type (robber or pollinator) across all visitors on each day of sampling. While this method fails to capture variation in the relative abundance of each visitor species, it is sufficient to describe the variation in visitation important to our question, specifically, the ratio of robbing to pollinating visits experienced by *C. caseana*. To control for differences in sampling effort, we used corrected visitation rate (visits observed per minute of sampling) as our response. We used a paired t-test to compare corrected pollinator and robber visitation rates with day as the unit of replication.

Pollen saturation hypothesis: How do single visits from pollinators affect stigmatic pollen loads?

We performed a single-visit pollen receipt study (31 July, and 4 and 5 August 2015) to estimate how many pollinator visits to *C. caseana* flowers are required to saturate stigmas with pollen and thus maximize female mating success, and to estimate autogamous pollen transfer rates. One to three days before each sampling date, we covered one haphazardly chosen stalk on each of three haphazardly chosen plants with bridal veil bags to prevent any floral visitation. Before bagging, any open flowers were removed, allowing us to use only virgin flowers. On each day of sampling, we removed the bag from stalks and immediately sampled two virgin flowers per stalk (*autogamy controls*), placing them in individually-labeled glassine envelopes and storing them on ice. These unvisited flowers allowed us to assess the amount of pollen that is transferred to the stigmas through autogamous self-pollination in the absence of pollinator visitation. We then observed the stalks until they were visited by a pollinator. Immediately after the pollinator departed, we collected two to four of the flowers visited and placed them in individually labeled glassine envelopes, storing them on ice. Each plant was only sampled once.

We measured single-visit pollen deposition from the flowers of 10 plants, and all pollinator visits were by *Bombus* spp. In the lab, we mounted stigmas on microscope slides using basic fuchsin gel (Kearns and Inouye, 1993) and counted all conspecific pollen grains under a compound microscope at $100\times$ magnification.

Stigma-pollen saturation (Ashman et al., 1993) occurs when a stigma receives at least one viable conspecific pollen grain per ovule, and thus serves as a proxy for the upper limit of female mating success. To estimate the number of pollen grains necessary to reach stigma saturation for *C. caseana*, we collected five to six ovaries from each of 13 *C. caseana* plants on 5 July 2016 (N = 77 total ovaries). We collected ovaries into 70% ethanol in the field. To fix the ovaries, we transferred them to 80% lactic acid for 48 h at room temperature. We then rinsed them with DI water and stored them in 70% ethanol until dissections. We counted the ovules in each ovary under a dissecting microscope by pulling the ovary walls apart using fine tip forceps which revealed the fixed ovules.

Statistical analyses

To assess single visit deposition, we used a paired t-test to compare the mean stigmatic pollen loads of virgin flowers within each plant with the means of the flowers that received a single pollinator visit. We qualitatively compared mean stigma pollen receipt from single pollinator visits to the number of ovules in flowers to assess the degree to which stigmas were saturated with pollen after a single pollinator visit.

DATA DEPOSITION

Data available from the Dryad Digital Repository: < <http://dx.doi.org/10.5061/dryad.bh6hs70> >

(Heiling et al. 2018).

RESULTS

Are the effects of nectar robbing on female function neutral?

As we predicted, using both observational and experimental approaches, we found no significant effects of nectar robbing on estimates of female function in *C. caseana*, suggesting that the effects of nectar robber were neutral.

I. Observational study

There was no difference in pollen tube count between unrobbed and robbed flowers ($\chi^2_1 = 2.81$, $P = 0.09$). Unrobbed flowers had 60.4 ± 2.7 (mean $\pm$ SE) pollen tubes per pistil, and robbed flowers had 57.6 ± 2.2 pollen tubes per pistil.

II. Experimental study

There was no difference in pollen receipt between experimentally robbed and unrobbed flowers ($\chi^2_1 = 4.39$, $P = 0.11$). Unrobbed flowers received 24.83 ± 0.96 (square-root transformed mean $\pm$ SE) conspecific pollen grains per flower per raceme, while flowers in the PR and CR treatments received 28.16 ± 1.01 and 28.54 ± 0.88 pollen grains, respectively. Likewise, we found no effect of experimental robbing on proportion fruit set ($\chi^2_2 = 2.05$, $P = 0.36$) or seed set per raceme ($\chi^2_2 = 0.37$, $P = 0.83$). Proportion fruit set in unrobbed plants was 0.74 ± 0.04 (mean $\pm$ SE), in PR plants was 0.73 ± 0.05 , and 0.83 ± 0.04 in CR plants. Mean seed set per raceme was 7.56 ± 0.83 (mean $\pm$ SE) for unrobbed plants, 6.73 ± 1.04 for PR plants, and 8.65 ± 1.64 for CR plants.

Non-discrimination hypothesis: Are nectar rewards diminished by robbing?

Within plants, approximately 15% more unrobbed flowers bore nectar than did robbed flowers ($t_{30} = 3.35$, $P = 0.002$) (Fig. 1.1a). Not only were robbed flowers more likely to be empty, they also had approximately half the amount of nectar on average relative to unrobbed flowers ($t_{30} = -4.77$; $P < 0.0001$) (Fig. 1.1b).

Non-discrimination hypothesis: Do pollinators discriminate between robbed and unrobbed flowers?

Our final model for the total number of bouts received by robbed and unrobbed pairs of stalks included robbing treatment and number of flowers per stalk as fixed effects and plant as a random effect for the conditional model, and a uniform probability of producing a structural zero in the final zero-inflation model. Our final models for the mean time spent per flower and the mean proportion of flowers visited per stalk included treatment as a fixed effect and plant as a random effect.

Over nearly 70 person-hours of observation, we observed 611 flower visits by pollinators, with 571 flower visits by *Bombus appositus*, 24 by *B. flavifrons*, 13 by *B. bifarius*, and three by an Andrenid species. Across all analyses, results suggest that pollinators did not discriminate between robbed and unrobbed stalks. There was no effect of robbing treatment on total bouts received per stalk ($\chi^2_2 = 0.78$; $P = 0.68$). Unrobbed stalks received 1.24 ± 0.13 bouts per stalk (mean $\pm$ SE) and robbed stalks 1.07 ± 0.15 bouts per stalk. We found no significant effect of robbing treatment on mean proportion of flowers visited per stalk ($\chi^2_1 = 0.27$, $P = 0.60$) and mean time spent per flower ($\chi^2_1 = 0.04$; $P = 0.84$). The mean proportion of flowers visited per stalk (ln transformed mean $\pm$ SE) was 1.47 ± 0.18 in the unrobbed treatment and 1.08 ± 0.15 in

the robbed treatment. Bees spent (mean $\pm$ SE) 0.08 ± 0.09 sec/flower in unrobbed flowers and 0.05 ± 0.6 sec/flower in robbed flowers.

Visitor prevalence hypothesis: How common are pollinator visits relative to nectar robber visits?

In 78 person-hours of observation (across 16 d), we recorded 9,709 individual flower visits. Pollinating species included *B. appositus*, *B. bifarius*, *B. flavifrons*, *B. nevadensis*, *B. rufocinctus*, and a small number of unidentified non-*Bombus* bees, with *B. appositus* being the most common pollinator (approximately 52% of all pollinating visits observed). Robbers included *Bombus bifarius*, *B. flavifrons*, *B. frigidus*, *B. kirbiellus*, and *B. occidentalis*, with the latter being the most common robber (approximately 34% of all robbing visits observed). Of the 9,709 total visits, 6,709 were pollinating visits and 3,000 were robbing visits, making pollinator visitation to *C. caseana* more than twice as common as robber visitation ($t_{15} = -4.1$, $P < 0.001$) (Fig. 1.2). This pattern was consistent both within days as well as across the dates sampled (Fig. 1.2). An analysis at the site level showed the same pattern as the global analysis (data not shown).

Pollen saturation hypothesis: How do single visits from pollinators affect stigmatic pollen loads?

We found that ovaries contained 4-12 ovules each (mean $\pm$ SE = 6.96 ± 0.18 ovules per ovary). Thus, stigma saturation in *C. caseana* should occur after the receipt of a fairly low number of viable pollen grains.

All of the *C. caseana* stigmas that we sampled bore copious amounts of pollen. Across virgin and single-visit flowers, conspecific pollen counts on *C. caseana* stigmas ranged from 1,706-11,692 grains, indicating that it is unlikely that these *C. caseana* populations are pollen

limited. Virgin stigmas bore large quantities of conspecific pollen (Fig. 1.3), suggesting autogamous self-pollination. The stigmas of virgin flowers had approximately twice as many pollen grains as stigmas of flowers that received a single visit from a pollinating bumble bee ($t_8 = 3.31$, $P = 0.01$) (Fig. 1.3).

DISCUSSION

Most studies of species interaction focus on interactions that yield mutually negative, mutually positive, or mixed positive and negative outcomes for the species involved (Goldberg and Barton, 1992; Hoeksema and Bruna, 2000; Sih et al., 1985), whereas commensalisms are poorly studied relative to their frequency in nature (Palumbi, 1985; Maloof and Inouye, 2000; Sáyago, et al. 2013). Although “exploitation” implies a negative interaction, some studies show that exploitation of pollination mutualisms can be commensal, possibly benefiting the exploiter at no cost (or benefit) to the plant (Fumero-Cabán and Meléndez-Ackerman, 2013; Morris, 1996). Here we also show that exploitation of nectar by robbing bees has neutral effects on female function of *Corydalis caseana*. These results are consistent with those of Maloof (2001) who showed thirteen years earlier in some of the same *C. caseana* populations that robbing was common but had no detectable effect on female function. Our goal here was to mechanistically explain why nectar robbers are commensals in this system. To understand the mechanisms driving these neutral effects, we empirically evaluated three non-mutually exclusive hypotheses, and found evidence consistent with each of them.

Non-discrimination hypothesis

While the lower reward levels we observed in robbed *C. caseana* flowers could serve as a motivation for pollinators to avoid them, we found no evidence that pollinators did in fact

discriminate against robbed flowers. These results are consistent with results in some other bee- and bird-pollinated systems (Fumero-Cabán and Meléndez-Ackerman, 2013; Rust, 1979). For example, in *Linaria vulgaris* (Plantaginaceae), robbing reduces nectar standing crop by >50%, but there is no evidence that *Bombus pascuorum* or *B. hortorum* pollinators discriminate between robbed and unrobbed flowers (Stout et al., 2000). Likewise, Rust (1979) found that artificially robbed *Impatiens capensis* (Balsaminaceae) flowers did not experience reduced pollination success relative to unrobbed flowers and found no evidence that bee pollinators differentiated between flowers on the basis of robbing status. Bee pollinators can make foraging decisions based on nectar availability (Pleasants, 1981). Why pollinators discriminate in some systems and not in others is not fully understood, though it may depend on underlying variation in reward levels within the plant species. For example, theoretical and empirical work addressing the occurrence of unrewarding flowers within otherwise rewarding plants suggests that, above some threshold reward frequency or concentration at the plant or population level, pollinators may not avoid unrewarding flowers because it is more energetically costly to do so than it is to visit them (Bell, 1986). That is, if variation in reward levels is high, whether they are robbed or not, avoiding robbed flowers may not increase foraging efficiency even if they do tend to contain less nectar. This may be especially true in systems where nectar production varies substantially throughout anthesis, and in systems in which flowers refill at a constant rate (as in *C. caseana*). Indeed, this could be the case in the *C. caseana* system, as nectar production continues throughout a flower's six- to seven-day lifetime. However, we limited our sample to flowers of roughly the same age (approximately one to two days post anthesis), and so were unable to test for any age-dependent differences in the relative nectar volumes of robbed and unrobbed flowers in this dataset. It is important to note, however, that in some cases, pollinators do discriminate

between robbed and unrobbed flowers, especially in bird-pollinated systems (Arizmendi et al., 1996; Irwin and Brody, 1998) and in some bee-pollinated systems (Dohzono et al., 2008; Zhang et al., 2014; Zimmerman and Cook, 1985). Understanding the proximate cues that pollinators use to perceive robbed flowers and the cognitive constraints they face may yield mechanistic insight into why they discriminate against robbed flowers in some cases and not others.

Visitor prevalence hypothesis

The ratio of robber visitation to pollinator visitation was low at the site and in the year that we conducted the study, consistent with the pollinator prevalence hypothesis. The timing of interactions between a species and its mutualists and exploiters is generally critical to determining the outcomes of those interactions (Barker and Bronstein, 2016). Thus, it is important to note not only the ratio of robber-to-pollinator visits, but also the timing of those visits relative to each other. We found that not only across days, but also within days, plants were more likely to be visited by a pollinator than a robber (Fig. 1.2). Additionally, we found no evidence that robbers and pollinators partitioned the day, though such partitioning has been observed in other systems (Rust, 1979). While the lack of discrimination by pollinators against robbed flowers in this system would likely dampen any potential effects related to the timing of interactions relative to one another, a similar lack of a temporal pattern in pollinator and robber visitation may be important to neutral effects of robbing on plant reproductive outcomes in other systems. Alternatively, for systems in which robber visits tend to occur before pollinator visits, and pollinators discriminate against robbed flowers, if neutral effects of robbing are observed, they would not be explained by the visitor prevalence hypothesis.

Robbing “frequency” is often estimated only by the number of robbed flowers per individual plant in a population. However, this is only a proxy for robber visitation frequency, especially when considering secondary robbing (since it leaves no evidence behind that it has taken place). Assessing the visitor prevalence hypothesis requires information on the actual frequencies of floral visits by robbers and pollinators. Counting robbing holes alone can over- or underestimate the prevalence of robbers in a visitor community. For example, Stout et al. (2000) found that while 96% of *Linaria vulgaris* flowers at their site in southern England had robbing holes, visits by nectar robbers only accounted for approximately 62% of all floral visits. Likewise, in two populations of *Impatiens capensis* in Delaware, USA, Rust (1979) found that up to 72% of flowers were robbed on 12 sampling dates, but robbers accounted for only half of all visitors observed. These results suggest that counting robbing holes in flowers may not be a sufficient way to assess the severity of robbing activity relative to other floral visitation, and that observing the real-time activity of pollinators and robbers is necessary to assess the visitor prevalence hypothesis.

Pollen saturation hypothesis

The pollen saturation hypothesis predicts that if plant reproduction is not pollen-limited, small to moderate reductions in pollinator visitation to nectar-robbed plants and flowers may not translate into differences in female reproductive success (Burkle et al., 2007). We found that all *C. caseana* stigmas that we examined were saturated with pollen, bearing pollen loads well in excess of the number of ovules available, consistent with the pollen saturation hypothesis. The high frequency of pollinator visits combined with the high autogamous self-pollination suggests that pollen limitation is unlikely in these populations of *C. caseana*. Indeed, working in some of

the same populations, Maloof (2000) found that open-pollinated *C. caseana* were not pollen limited, as measured by pollen-supplementation experiments.

Surprisingly, stigmas of *C. caseana* were saturated with pollen even without pollinator visitation, suggesting that *C. caseana* flowers receive significant amounts of autogamous pollen. *Corydalis caseana* anthers are positioned in contact with the stigma (Maloof, 2000) and shed pollen directly onto the stigma upon dehiscence or manipulation of the corolla; thus, autogamous pollen transfer could explain the universally high stigmatic pollen loads that we observed. As copious self-pollen is shed directly onto stigmas in *C. caseana*, autogamous pollen transfer may represent a bet-hedging strategy for this self-compatible species. Such a strategy may result in reduced outcrossing in exchange for some insured fertilization at low visitation frequencies. Yet, *C. caseana* receives relatively high pollinator visitation rates (here and Maloof, 2000). Thus, it is not clear whether such a bet-hedging strategy would help or hinder outcrossed pollination, warranting additional investigation of *C. caseana* reproductive biology.

Interestingly, stigmas from flowers visited once by pollinators bore a mean of 3838 ± 273 (SE) pollen grains while stigmas from virgin (unvisited) bore approximately twice as much, with a mean of 6651 ± 774 (SE) grains. This result suggests that, for at least the first few visits to a flower, pollinators remove large numbers of self-pollen grains from stigmas. Thus, the stigma may serve dual roles as both a pollen receiving organ and a secondary pollen presentation surface in *C. caseana*, similar to other stylar presenters, such as in some members of the Marantaceae and Polygalaceae (e.g., Howell et al., 1993). It is likely that there is a threshold of pollinator visits that must occur before outcross pollen is able to gain access to the stigmatic surface relative to self-pollen. One limitation to the interpretation of our results is that we did not emasculate flowers prior to measuring single-visit pollen deposition (as in Gerber, 1985); thus,

the pollen we counted is a mixture of outcrossed and self-pollen, and we do not know the exact number of pollen grains a visitor deposits in a single visit. Repeating this single-visit pollen deposition study using emasculated flowers would yield additional ecological insight, though conducting such a study using emasculated flowers would be exceptionally challenging given the fusion of the androecium and gynoecium.

Nectar robbing as a commensal species interaction

Here we demonstrate multiple avenues through which the effects of nectar robbing on the fitness of robbed plants can be mitigated. These buffers allow an apparently antagonistic interaction to function in nature as a commensalism. At least three areas of further work are important to understanding nectar robbing commensalisms. First, in cases where robbers do not damage the reproductive or nectar-producing structures while robbing, the sign of the plant—nectar robber interaction should be context dependent. Inter- and intra-annual variation in the relative frequencies of robber and pollinator abundance are common in plant-pollinator-nectar robber systems (Irwin and Maloof, 2002). For example, *B. occidentalis* accounted for only about 1.2% of all floral visits by *Bombus* species (120 out of 9,709 floral visits) to *C. caseana* in our 2014 data; compare this to the 30% *B. occidentalis* visitation rate (63 of 205 total visits) observed by Maloof in some of the same sites in 1996 (Maloof, 2000). Such variation could cause the extent to which a plant population experiences robbing to fluctuate dramatically between years and locations, and these fluctuations could cause the magnitude of any robbing effects to vary accordingly. The sign and magnitude of such effects should depend both on the degree to which pollinators in the system discriminate against robbed plants, and how sensitive the plant species is to pollen limitation. However, surprisingly few studies have measured spatio-temporal

variation in the fitness effects of nectar robbing in plant-robber-pollinator systems, and so we do not know how commonly the interaction shifts among commensalism, antagonism, and mutualism. Second, we focused on measuring the effects of robbing on female components of plant reproduction, but most flowering plants are hermaphrodites. Assessing the degree to which the effects of robbing are neutral for plants requires the measurement of both female and male components of plant fitness. While in some cases species interactions can have similar effects on male and female function (reviewed in Schaeffer et al., 2013), in other cases species interactions can promote sexual conflict, driving strongly divergent effects on the two sexual functions (e.g., Zhang et al., 2009). Because the effects of nectar robbing often manifest through effects on pollinator behavior, and because male function can be more sensitive to pollinator behavior than is female function (e.g., Stanton et al., 1986), measuring both sexual functions is critical for fully assessing the degree to which robbing affects plant reproduction. Including measurement of male function may also require the development of additional hypotheses to explain neutral effects. Third, it remains unresolved how nectar robbers should be included in networks of interactions between plants and floral visitors, especially in cases where the interaction is commensal, as the effects of nectar robbers may be quite weak overall. Weak interactions can be important in stabilizing food webs (McCann et al., 1998), and weak and commensal interactions between plants and robbers may be important in promoting community stability and persistence in plant-floral visitor webs. However, the degree to which this occurs is unknown and warrants further research.

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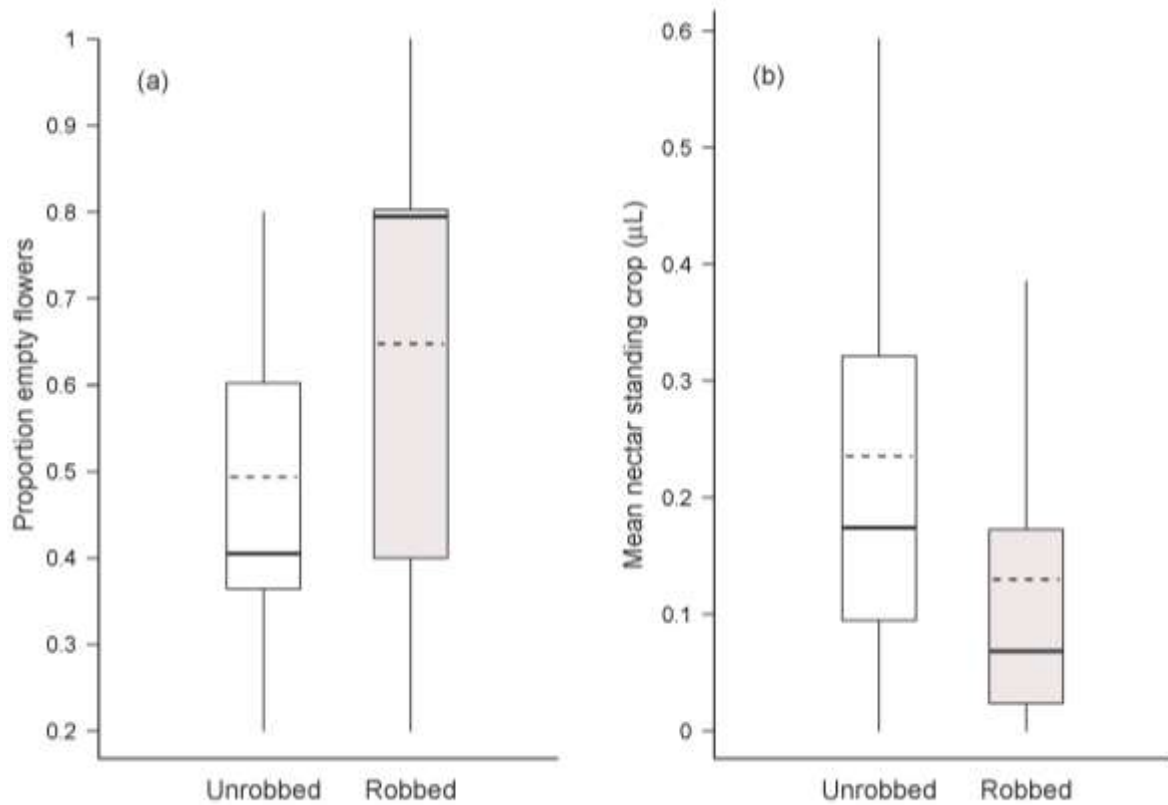


Figure 1.1. Nectar robbing was associated with reduced nectar rewards. (a) Robbed flowers were more often empty of nectar than were unrobbed flowers on the same plant, and (b) robbed flowers had lower nectar standing crops than unrobbed flowers on the same plant. Bars are boxplots, with lower and upper ends of boxes depicting the lower and upper quartiles, respectively. Solid bands indicate medians, dashed bands indicate means. Whiskers extend across the data range, excluding outliers. N = 31 plants.

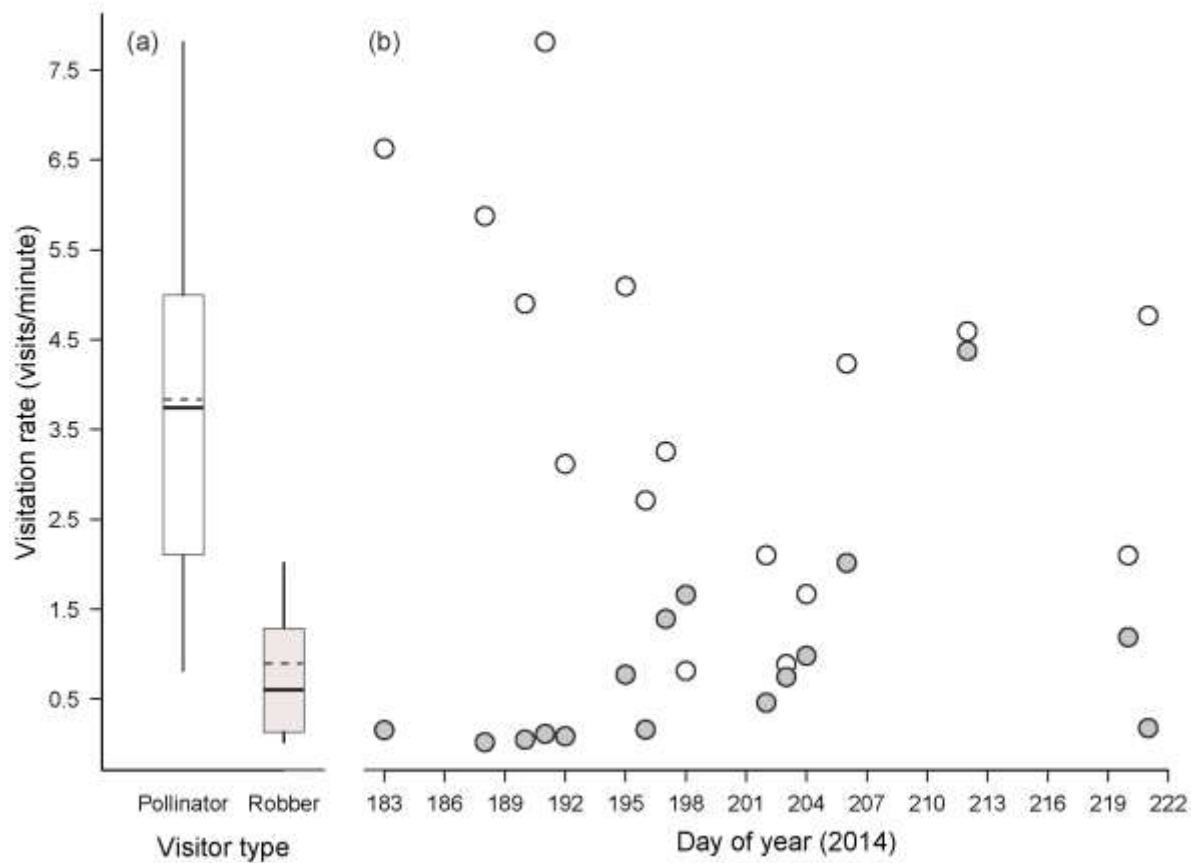


Figure 1.2. Visitation rates (standardized by sampling effort) by pollinators and nectar robbers in a *Corydalis caseana* population across 16 days of observation. To standardize visitation rate by sampling effort, we divided the number of visits of each type (pollinator or robber) observed each day by the effort (minutes) spent on observations that day. Pollinator visits were more than twice as common as robbing visits. (a) Boxes are box plots depicting quartiles, median, mean, and data range as in Figure 1. (b) Points are paired visitation rates for each day of sampling; empty points are pollinator visitation rates and gray points are robber visitation rates. N = 16 days of sampling.

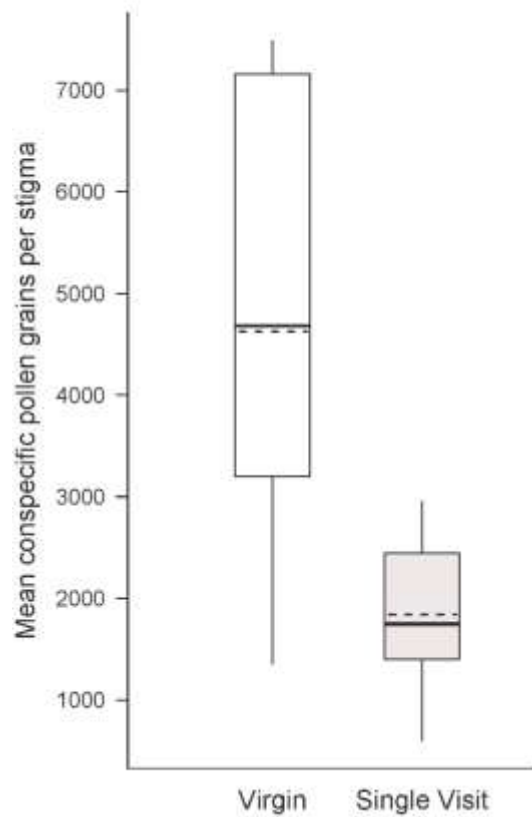


Figure 1.3. Virgin, unvisited flowers of *Corydalis caseana* had nearly twice as many pollen grains as flowers on the same plant that received a single visit by a pollinating *Bombus* spp. Boxes are box plots depicting quartiles, median, mean, and data range as in Figure 1. N = 9 plants.

CHAPTER 2: The Pollen Reward Paradox – A Strategy for Minimizing the Cost of Pollination?

Jacob M. Heiling, Judith L. Bronstein, and Rebecca E. Irwin

ABSTRACT

1. Pollen is often used as a floral reward. Yet, pollen lost to consumption by pollinators represents lost reproductive opportunities for plants. While pollen's importance as a floral reward is widely appreciated, the use of pollen as a reward presents a paradox. Pollen rewards require plants to pay a price in lost pollen to move the remainder of their pollen to recipient flowers. Some plants are strictly pollen rewarding and make no nectar or other rewards. It is unknown what benefits the pollen-rewarding strategy may confer to plant fitness relative to employing other reward strategies, such as offering nectar or nectar and pollen.

2. We compared pollinator forager behavior and plant reproductive success of strictly pollen-rewarding plants to those with pollen and nectar rewards. To do so, we added artificial nectar to silvery lupine (*Lupinus argenteus*) individuals to simulate a novel nectar and pollen-rewarding phenotype in this strictly pollen-rewarding species and compared pollinator foraging behavior and female reproductive success between these nectar-added and nectarless control plants.

3. Pollinators collected both pollen and nectar from nectar-added plants and spent 27% longer per flower on nectar-added than on control plants. Single visit pollen deposition increased with visit duration. However, the differences in pollinator behavior and pollen deposition in response to nectar addition did not affect female reproductive success relative to control plants. We suspect this is the case because we found no evidence that plants were pollen-limited in the study

population, which may explain why changes in pollinator behavior and pollen deposition did not translate into differences in plant reproduction.

4. *Synthesis*. We found no evidence that having a nectar reward would provide any additional benefits to reproduction in a pollen-rewarding plant. These results suggest that the pollen-rewarding strategy may allow plants to minimize investment in nectar rewards without compromising their attractiveness to pollinators and subsequent female plant reproduction.

INTRODUCTION

Many plant—animal mutualisms involve the exchange of rewards (e.g., nectar, fruit, nesting sites, etc.) for services (e.g., pollen dispersal, seed dispersal, and protection). In these mutualisms, animal partners act as consumers and host plants as resources (Holland et al., 2005). Natural selection should favor hosts that are able to maximize their benefits while minimizing the costs associated with providing rewards to their partners (Bronstein, 2001). Costs of rewards may be tied to resource allocation, mating opportunities, or both.

Nectar and pollen are the two most common floral rewards (Simpson and Neff, 1983). Of the two, nectar is the most widespread reward flowering plants provide to their mutualist pollinators, but it is not the ancestral condition (Simpson and Neff, 1983; Armbruster, 2012). Pollen is thought to have been the first floral resource that insects would have visited flowers for and, thus, served as the first pollinator reward (Simpson and Neff, 1983). Some angiosperm species are strictly nectar rewarding, while others have maintained or secondarily derived a strictly pollen based reward strategy or offer both pollen and nectar rewards. In some cases, examples of all three of these reward strategies can be found in the same plant families and even genera (e.g. Fabaceae, Orobanchaceae, Papaveraceae, and Solanaceae, among others; Simpson and Neff, 1983; Macior et al., 2001; Etcheverry et al., 2012).

The use of pollen as a sole reward for pollinators presents 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 plants that offer nectar and pollen (Simpson and Neff, 1983; Etcheverry et al., 2012), presumably to offset lost reproductive opportunities due to pollen consumption (Darwin, 1859). Why should a plant pay a direct reproductive cost by offering

pollen as a reward? While the pollen-reward strategy is widespread, we know relatively little about the benefits of this strategy. Do pollinator behaviors differ between strictly pollen-rewarding plants and plants that offer both nectar and pollen as rewards? If so, do any such differences result in higher reproductive success in pollen-rewarding plants, offsetting the cost of providing pollen as a reward? Until we better understand how this reward strategy mediates pollinator behavior, and how changes in pollinator behavior influence plant reproductive success in these species, it will remain difficult to interpret why some lineages have maintained or re-derived this character state while others (often close relatives) have not.

The goal of this study was to discover whether fitness benefits differ between the pollen-only and pollen + nectar reward strategies, and whether such differences can be explained, in the absence of differences in resource allocation costs, by differences in forager behavior. We did so by comparing the behavior of pollinators and plant reproduction on natural pollen-rewarding plants to plants that were treated with artificial nectar rewards. We performed an experiment in which we added nectar to a subset of *Lupinus argenteus* plants to simulate a novel nectar and pollen rewarding phenotype in a population of this strictly pollen-rewarding species (Gori, 1989). One of the benefits of this experiment is that it allowed us to assess the effects of nectar addition on pollinator behavior and plant reproduction independent of the potentially confounding effects of variation in resource investment. While the mating cost imposed by pollen consumption is a cost to reproductive success through male function, benefits associated with this strategy may come through male function, female function, or both (Stanton et al., 1986; Wilson et al., 1994). Due to the immense difficulty of reliably assessing reproductive success through male function in the field (Snow & Lewis, 1993), we focused our direct measurements of plant reproduction through female components of reproduction 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.

Specifically, we asked: (1) How does nectar addition to pollen-rewarding plants affect pollinator foraging behavior and host-plant fitness? Finding a significant, positive effect of nectar addition on per-flower visit duration but not on female reproductive success, we then asked: (2) How does time spent per flower affect single-visit pollen deposition in *L. argenteus*, and (3) Does *L. argenteus* exhibit pollen limitation?

METHODS

Study System

Lupinus argenteus Pursh (Fabaceae) is a long-lived herbaceous perennial that occurs 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 Gunnison County, Colorado, USA near the Rocky Mountain Biological Laboratory (RMBL). The flowers of *L. argenteus* are blue to purple with white banner spots, papilionaceous, and are borne on indeterminate stalks. Plants produce from 1 to over 40 flowering stalks at a time (median 4 stalks per plant) with 1-20 flowers per stalk (median 6 flowers per stalk), with a single plant producing anywhere from a few to nearly 1000 flowers in a season (plants at our site produced a mean of 300 flowers/season). *Lupinus argenteus* is strictly pollen-rewarding – like most if not all other lupines – and bears no floral nectaries. It is self-incompatible and pollinated by pollen foraging bees, primarily in the genera *Bombus* and less

commonly by *Andrena* spp. and *Megachile* spp. (Dunn, 1956; Gori, 1989; Harder, 1990). Pollen is dispensed from a pore in the distal end of semi-fused keel petals (Müller, 1883; Dunn, 1956; Harder, 1990) which must be depressed away from the axis formed by the banner and petiole to eject pollen. Bees activate this “piston” like mechanism (Müller, 1883; Dunn, 1956) by bracing their heads on the banner petal, clutching the wing petals that enclose the keel with their mid or fore legs, and extending their legs (Dunn, 1956; Gori, 1989; Harder, 1990). Pollen is subsequently deposited on their abdomen from the keel pore (Dunn, 1956; Gori, 1989; Harder, 1990). Some bees, such as megachilids, may circumvent this mechanism by “unzipping” the semi-fused keel petals and scraping pollen directly from the anthers with their midlegs (*Pers. obs.*).

Experimental Design

Question 1: How does nectar addition to pollen-rewarding plants affect pollinator foraging behavior and host-plant fitness?

We selected 98 individual *L. argenteus* at a single site (“Bellview Bench”, N: 39.00490°, W: -107.03182°, 10,301 masl, approx. 11×11 m). Plants were randomly assigned to one of two treatments: nectar addition (pollen plus nectar rewarding) or control (pollen rewarding only), with 49 plants per treatment. Treatments began soon after flowering (on 14 July 2016) and ended once plants had passed peak bloom (13 August 2016). We applied treatments daily to all open flowers on each plant.

We deposited 2 µl of artificial nectar (40% v/v sucrose) between the keel and wing petals of all open flowers on each of 49 plants. Nectar was added using a repeating micro-pipette (Fisherbrand, HandyStep). We added 2 µl of 40% v/v sucrose per flower to mimic the

concentrations and nectar standing crops in the co-flowering community (Luo et al., 2014). On 49 control plants, we probed all open flowers with a dry micro-pipette tip to control for flower handling. Between plants, the micro-pipette tip was cleaned with ethanol to avoid transferring pollen. The study site was sheltered and at high elevation, so plants were heavily dew laden in the early morning. To avoid diluting our nectar with dew, we applied the nectar addition once flowers were dry, approx. 10:00-11:00AM. On all plants daily, we recorded the number of open flowers on each individual (floral display size) but did not mark flowers.

Pollinator visitation

We began pollinator observations on 19 July 2016, 5 days after treatments were started. We performed observations on 14 days between 19 July and 4 August 2016. Observations were conducted for 1 hour per day immediately following nectar application. We limited our observations to 1 hour per day because pilot trials showed that our artificial nectar began to evaporate rapidly in *L. argenteus* flowers in field conditions after that period of time. We followed each bee that we observed visiting our experimental plants, recording the number of visits that each plant received, a proxy for relative mating success (Stanton et al., 1986; Engel and Irwin, 2003; but see: King et al., 2013); flowers probed per-plant per-visit, serving as a proxy for both female mating success and the potential for pollen discounting through geitonogamous pollen-transfer (Klinkhamer and de Jong, 1993; Klinkhamer et al., 1994); and the per-flower visit duration, which is often positively correlated with pollen deposition rates (especially in plants visited by bumble bees; King et al., 2013; Thøstenes and Olesen, 1996) and can serve as a proxy for pollinator effectiveness. We also recorded the species and caste of each visitor. If the addition of nectar affects the positioning of bees within flowers, this could affect

the placement of pollen on bees' bodies and their potential to act as pollen vectors between nectar addition and control plants (e.g., Tong et al., 2018). However, because we found this to be stereotyped on *Lupinus* spp., and consistent with that described by other authors (Dunn, 1956; Harder, 1990), regardless of the presence or absence of artificial nectar during pilot trials, we did not record body position in our study.

Plant reproduction

We discontinued nectar addition on 14 August 2016 when plants had passed peak bloom. We collected all fruit-bearing stalks between 29 August and 4 September 2016, allowing fruit to dry on the stalk before dissecting them to collect and count the number of mature and aborted seeds in each fruit. We also weighed the seeds produced per plant to the nearest 0.1 mg.

Statistical analysis

We conducted all analyses (here and below) in R version 3.5.1 (R Core Team, 2018). We used the base R package 'stats' to fit linear models (LM) (R Core Team, 2018); the 'MASS' package to fit generalized linear models (GLM) (Venables, 2002); and the 'lme4' package to fit linear and generalized mixed effects models (LMM and GLMM, respectively) (Bates, 2010).

Pollinator visitation

We analyzed three metrics of pollinator visitation: (1) the total number of visits each plant received (approaches that resulted in flower visitation), (2) the number of flowers probed per-plant per-visit, and (3) the time spent per flower. We assessed the effect of nectar treatment on the total number of pollinator visits that each plant received across all observation days using a generalized linear model with a negative binomial error distribution (visit data were

overdispersed, dispersion parameter = 2.04; $\theta = 1.602$, fit by maximum likelihood) with nectar treatment as our main predictor, mean floral display size (mean number of flowers open per individual per day across the entire season) as a covariate, and the interaction between nectar treatment and floral display size. The interaction was not significant, and so we removed it from our final model.

To assess the effect of nectar treatment on the number of flowers probed per plant visit, we fit a Poisson GLMM with nectar treatment, floral display size (on the day a visit was observed), and the interaction between nectar treatment and floral display size as fixed effects in the initial model. The interaction was not significant, and we dropped it from our final model. We included as random effects visitor ID (to account for resampling of individual plants within days and across the season) and date nested within individual plant. We used the R package ‘lmerTest’ (Kuznetsova et al., 2015) to perform inference tests for GLMMs. To reduce Eigenvalue size and improve model fit, we scaled display size using the base R function *scale()*.

We used an LMM to compare the time visitors spent on each flower that they visited on nectar addition and control plants. In the initial model, we used the natural log transformed mean flower visit duration as the response with nectar treatment (addition vs. control), floral display size, and their interaction as fixed effects with an interaction term, and visitor ID and date nested within individual plant as random effects. The interaction was not significant, and so we removed it from our final model. We used the R package ‘lmerTest’ (Kuznetsova et al., 2015) to perform inference tests for LMMs.

Plant reproduction

We used four metrics to estimate female plant reproductive success: (1) total seed production (total number of mature seeds produced by each individual plant), (2) mean seed set per fruit per plant, (3) mean seeds aborted per fruit per plant (mean number of ovules that initiated but did not make mature seeds in each fruit on each plant), and (4) mean weight per seed per plant. Total seed production simultaneously accounts for both pollen and resource limitation, both of which can affect plant investment in seeds. Whereas mean seeds produced per fruit provides an index of visitation rates by pollinators and pollination quality on a per-flower basis. Both seed abortion and mean seed mass can be affected by pollen quality (Levin, 1984; Heschel and Paige, 1995; Vaughton and Ramsey, 1997). Because we did not mark individual flowers and *L. argenteus* flowers that do not make fruits do not leave reliable scars on the stalk (scars from flowers that never open are identical to scars from flowers that opened), we could not calculate proportion fruit set.

To compare the effect of nectar treatment on total seed production per plant, we fit a negative binomial GLM (seed count data were overdispersed, $\theta = 0.765$, fit by maximum likelihood) with total seed production as a response and nectar treatment (addition vs. control), mean floral display size (mean display size per plant across the flowering season), and their interaction as fixed effects. This interaction was not significant, and so we removed it from the final model. Shapiro–Wilk tests revealed that seeds per fruit ($W = 0.920$, $P < 0.0001$), aborted seeds per fruit ($W = 0.963$, $P = 0.029$), 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 numbers of mature and aborted seeds per fruit per plant and mean mass of mature seeds using two-sample Wilcoxon tests (R function `wilcox.test()` in the ‘stats’ package).

Nectar removal

Although lupine flowers do not produce nectar (Müller, 1883; Dunn, 1956; Gori, 1989), bees probe the flowers with their glossa while pollen collecting (Farrer, 1872; Dunn, 1956; J. Francis, *personal communication; personal observation*). This may be a compensatory reflex resulting from the strain associated with the body and leg extension required to operate the floral mechanism (Dunn, 1956). Because of this stereotyped movement, bees were able to find our artificial nectar rewards easily. However, to verify that foragers responded to our artificial nectar by removing it, we applied artificial nectar to all open flowers on separate *L. argenteus* individuals from our focal plants (one individual per day on three days in July 2017) and waited for visitors to approach. When a bee initiated a visit on a test stalk, we allowed them to visit several flowers, but shooed them away before they revisited any flowers or visited all flowers on the stalk. We then used 5 μ L 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.

Statistical analysis

We used a paired t-test to compare the within individual mean nectar volumes of visited and unvisited flowers. To limit the confounding effect of evaporation, we abandoned stalks that were not visited within 30 mins post treatment. We did not include individual plant as a random effect in a mixed effects model because (1) we did not have enough data for a mixed effects model, and (2) such a model structure was not necessary as we were not comparing the response of foragers to different individuals, stalks, or flowers.

Question 2: How does time spent per flower affect single-visit pollen deposition in L. argenteus?

To determine whether pollinator effectiveness changed with per-flower visit duration in *L. argenteus*, we compared single-visit pollen deposition (SVD), a component of pollinator effectiveness (King et al., 2013), on nectar-addition and control inflorescences. We did so using the interview-stick method described by Thomson (1988). In brief, we affixed virgin *L. argenteus* inflorescences to the end of a 1 m rod, treated the flowers with either 2 or 4 μ L of artificial nectar (40% v/v sucrose) or probed the flowers with a dry pipette (control). We included the 4 μ L artificial nectar treatment to maximize variation in per-flower visit duration. Inflorescences were then offered to floral visitors (primarily *Bombus* spp.) that were foraging on *L. argenteus* in our site. When a bee accepted a flower, we recorded the duration of the visit using digital voice recorders and shooed the bee away when it exited the first flower that it accepted. We then removed the stigma from that flower with fine-point forceps and stored it in an individual, clean 1.5 mL vial on ice. In the lab on the day of stigma collection, we mounted stigmas on glass microscope slides using basic fuchsin gel (Kearns and Inouye, 1993), and we counted the number of conspecific and heterospecific pollen grains at $400\times$ under a compound microscope. We ran interview-stick trials on 3 days between 28 July and 12 August 2017 and counted pollen on a total of 24 stigmas. Heterospecific pollen deposition was low; out of 24 stigmas, only three received heterospecific pollen, with < 3 grains on each. As a result, we did not include heterospecific pollen in our analyses.

Statistical analyses

To assess the effect of visit duration on pollinator effectiveness (measured as single-visit pollen deposition), we fit a negative binomial GLMM (pollen count data were overdispersed,

dispersion parameter = 2.62; θ = 3.098, fit by maximum likelihood) with visit duration, nectar treatment (2 and 4 μ L 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 plants. To determine whether we could detect an effect of nectar volume on visit duration in this experiment, we also fit an LMM with visit duration as the response and nectar volume (0, 2, or 4 μ L) as a fixed effect and plant ID as a random effect.

*Question 3: Does *L. argenteus* exhibit pollen limitation?*

The magnitude of the effects of plant traits, such as the type of floral rewards offered, that influence pollinator behavior and plant reproduction should be more pronounced in pollen limited populations than in those that are not pollen limited. To evaluate whether our study population of *L. argenteus* experiences pollen limitation, we performed a hand-pollination experiment at the Bellview Bench site in 2017.

On July 7 2017, we marked 50 *L. argenteus* individuals and randomly assigned one-half to receive pollen supplementation and the other half served as controls (N = 25 plants per treatment). For plants in the pollen supplementation treatment, beginning at the onset of flowering and continuing until senescence (11 July-17 August 2017), we hand-pollinated all open flowers every third day to ensure that we were treating flowers during their approx. 96 hour period of stigma receptivity (Gori, 1989). We used pollen mixed from 5 individuals (10-20 flowers each, collected into a clean plastic vial) which were situated 10-20 m away from experimental plants to reduce the chances of both bi-parental inbreeding and outbreeding depression (Waser and Williams, 2001). To ensure that we were using viable pollen, we limited our pollen collection to flowers with white banner-spots from the terminal or sub-terminal whorl

in anthesis; pollen viability begins to decline in *L. argenteus* after approx. 48 hours 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 a visible dab of pollen onto the stigma using a size 3 camel hair paintbrush. To control for the effects of flower handling, we manipulated the flowers of control plants with forceps during counting but did not add pollen. On both pollen-supplemented and control plants, we counted each treated flower and used a fine point, felt tipped, oil-based paint pen to apply a small dot of blue paint to the adaxial surface of the calyx to prevent re-treating/re-counting flowers on subsequent days.

Upon terminating hand pollinations, we enclosed the stalks of all experimental plants in bridal veil bags to prevent seed loss due to explosive dehiscence during fruit ripening and herbivory by deer and marmots. We harvested plants when all of their fruit were ripe or nearly ripe (when fruits dried and became brittle), and we counted the mature and aborted seeds produced per fruit per plant and weighed seeds to the nearest 0.1 mg.

Statistical analysis

Because we were able to mark each flower produced on each focal plant in this experiment, we could measure proportion fruit set, which provides a metric of plant reproduction tied to pollination success. Thus, we assessed the effects of hand pollination on 5 metrics of female plant reproduction in this experiment: (1) proportion fruit set (number of seed-bearing fruits divided by the total flowers produced per plant); (2) total seeds produced per plant, (3) mean number of seeds produced per seed-bearing fruit; (4) mean number of seeds aborted per fruit, and (5) mean seed mass.

To assess the effect of hand pollination on proportion fruit set and total seeds produced per plant, we initially fit linear models with treatment (hand-pollination vs. control) as our predictor with total number of flowers produced (to test for effects of plant size or resources) as a covariate, along with their interaction. Neither the interaction term nor the total number of flowers were significant in either model and we dropped them from the final models. Both models passed Durban Watson and studentized Breusch-Pagan tests for residual autocorrelation and heteroskedasticity, respectively. Shapiro-Wilk tests revealed that the seeds per fruit ($W = 0.907$, $P = 0.004$) and aborted seeds per fruit ($W = 0.924$, $P = 0.011$) data were not normally distributed. Accordingly, we assessed the effects of supplemental hand-pollination on the mean numbers of mature and aborted seeds per fruit per plant and mean mass of mature seeds using two-sample Wilcoxon rank sum tests, as described above. We used a two-sample, unpaired t-test to assess the effects of supplemental hand-pollination on mean seed mass.

RESULTS

Question 1: How does nectar addition to pollen-rewarding plants affect pollinator foraging behavior and host-plant fitness?

Pollinator visitation. We observed 170 approaches resulting in visits to experimental plants, totaling 2,947 individual flower visits over 14 hours of observation. We observed 8 taxa of bees foraging on the focal plants, five identified to the species level (*Bombus appositus*, *B. bifarius*, *B. californicus*, *B. flavifrons*, and *B. mixtus*) and four identified to the genus level (*Andrena* sp., *Megachile* sp., and *Osmia* sp.). The most common visitors were *Bombus bifarius*, *B. flavifrons*, and *Megachile* sp., accounting for 51%, 35%, and 7% of all approaches resulting in visits, respectively. Among the *Bombus* sp., visitors were predominantly workers, with only one

queen *Bombus bifarius* and one queen *B. flavifrons* (out of 57 and 42 individuals, respectively) appearing in our observations.

Bees responded to nectar-addition plants by increasing the mean time spent per flower. Pollinators spent approx. 27% longer per-flower on nectar-addition than on control plants ($t_{40.27} = 3.832$, $P < 0.001$; Fig. 2.1). However, there was no difference in the number of times plants were visited (control: 1.510 ± 0.302 (SE) total visits/bouts, nectar-addition: 1.979 ± 0.313 (SE) total visits/bouts; $t_{95} = 1.152$, $P = 0.252$) or in the number of flowers probed per plant per visit (control: 18.73 ± 2.69 (SE) flowers per plant per visit, nectar addition: 16.12 ± 1.65 ; $Z = 1.100$, $P = 0.271$).

There was a significant effect of the covariate, mean floral display size, on some of the metrics of pollinator visitation. There was a significant positive effect of 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 ($t_{54.11} = 1.824$, $P = 0.074$). For all three metrics of pollinator visitation, the interaction between nectar treatment and mean display size was not statistically significant and was removed from each of the models.

Plant reproduction. Although nectar addition resulted in increased per-flower visit duration, we found no effect of nectar addition on any metric of plant reproduction, including number of seeds produced per plant ($Z = -0.854$, $P = 0.393$), number of mature (Z = -1.3729, $P = 0.085$) and aborted seeds per fruit ($Z = -0.1377$, $P = 0.445$), or mean seed mass ($Z = -0.515$, $P = 0.303$; Table 1). The covariate in the seeds per plant model, mean floral display size, had a

significant positive effect on total seed production (Estimate = 0.028 ± 0.004 , $Z = 7.465$, $P < 0.0001$; Fig. 2.2).

Nectar addition

Bees responded to nectar addition by collecting nectar in addition to pollen. We observed 21 individual foraging bouts on test stalks. We recovered, on average, 0.96 ± 0.19 μL less nectar (out of the 2 μL applied) from visited than from unvisited flowers in our nectar-removal trials (paired t-test: $t_8 = -4.89$, $P = 0.001$; Fig. 2.3).

Question 2: How does time spent per flower affect single-visit pollen deposition in L. argenteus?

Per-flower visit duration had a statistically significant positive effect on single-visit deposition of conspecific pollen (Estimate = 0.178 ± 0.051 SE; $Z = 3.493$, $P < 0.0005$; Fig. 2.4). However, nectar volume (0, 2, or 4 μL per-flower) did not have a statistically significant effect on visit duration in these trials ($Z = 0.238$, $P = 0.812$).

Question 3: Does L. argenteus exhibit pollen limitation?

We found no evidence that the plants in our study population were pollen limited. We found no effect of supplemental hand-pollination on proportion fruit set ($F_{1,37} = 1.240$, $P = 0.273$), total seeds produced per plant ($F_{1,37} = 0.102$, $P = 0.752$), mean seeds produced per fruit ($Z = 0.107$, $P = 0.543$), number of seeds aborted per fruit ($Z = 1.585$, $P = 0.944$), or mean seed mass ($t_{30.4} = -0.671$, $P = 0.507$; Table. 2).

DISCUSSION

While visitor behavior varied between nectar-added and control plants, this variation was limited to a single metric of forager behavior and did not result in a measurable effect on plant reproductive success through female function. Despite the additional reward available in the nectar-added plants in our study, the presence of nectar only increased per-visit duration, not the total number of visits or flowers probed per visit. Thus, offering a nectar reward did not make individuals in this species, which is already visited for pollen, any more attractive to its pollinators. Though pollinator effectiveness (measured as single-visit pollen deposition) increased modestly with per-flower visit duration, per-flower visit duration was higher on nectar-added plants. In general, the effects of visit duration on pollen deposition, and thus female function are mixed (King et al., 2013), but, consistent with our observations of SVD in this system, pollen deposition often does increase with visit duration in *Bombus* pollinated plants (Thøstesen and Olesen, 1996; Kudo, 2003; King et al., 2013). However, these effects did not translate into differences in female components of plant reproduction. This may be explained by the fact that the *L. argenteus* population we studied was not pollen-limited. However, pollen-limitation can vary in space and time (Campbell, 1987; Price et al., 2005; Vanhoenacker et al., 2006). Thus, in years or areas when pollinator visitation is less common, either due to variation in pollinator demography or competition with co-flowering species, increased visit duration could help prevent pollen limitation in this species. While the effect size of visit duration on pollen deposition that we observed was modest, small differences in pollen deposition could mean the difference between full seed-set and a failure to reproduce for these plants which bare relatively few ovules per ovary (4-9 in close relatives *L. arizonicus* and *L. sparsiflorus*;

Wainwright, 1978). However, there is no evidence that nectar rewards would provide any benefit to reproductive success in *L. argenteus* populations that do not experience pollen limitation.

Interestingly, our results contrast with those of the only other study of which we are aware that has attempted to assess the effects of nectar addition on pollinator behavior and plant reproduction in a pollen-rewarding species. Tong et al. (2018) described negative effects of nectar addition on plant reproduction in the pollen-rewarding *Pedicularis dichotoma* (Orobanchaceae). These negative effects stemmed from altered body positioning in bees foraging on nectar-added inflorescences, resulting in a failure of bees to contact anthers and stigmas (Tong et al., 2018). This may be, in part, due to the asymmetric floral architecture of the *P. dichotoma* flowers, requiring alternative handling techniques for pollen and nectar extraction, respectively. However, *L. argenteus* have bilaterally symmetrical papilionaceous flowers that do not require bees to handle them in a different manner than that required for pollen collection. Another interesting difference between the results of the two studies is the increased visitation frequency observed by Tong et al. (2018) to nectar-added flowers, and the absence of such an effect in our study. There are at least two, non-mutually exclusive, explanations for this difference. First, there may have been lower nectar availability or concentration in the co-flowering community of *P. dichotoma* compared to the *L. argenteus* co-flowering community, resulting in a higher relative value of the artificial nectar rewards in *P. dichotoma* compared to *L. argenteus*. Second, there may have been differences in the cognitive abilities, energetic demands, colony resource requirements, or other factors affecting foraging decisions between the pollinators of *P. dichotoma* (*Bombus friseanus*) (Tong et al., 2018) and *L. argenteus* (*Bombus appositus*, *B. bifarius*, *B. californicus*, *B. flavifrons*, *B. mixtus*, *Andrena* sp., *Megachile* sp., and *Osmia* sp.). It is unlikely that the observed differences in visitation were due to the volume or

concentration of artificial nectar, number of plants that nectar was added to, plant population size, elevation, or the duration of observations, as all of these factors were comparable between the two studies.

Two limitations of our study are the fact that we did not measure the effects of nectar addition on reproductive success through male function and we did not systematically vary floral display size. However, some of our results may hold implications for the role of floral display size on male mating success in plants that employ pollen only vs. pollen + nectar reward strategies. The increased time spent per flower on nectar supplemented plants may affect male reproductive success in two ways.

First, pollen removal typically increases with visit duration (Harder, 1990; Young and Stanton, 1990; Kudo, 2003). However, the relationship between per-visit pollen removal and male mating success is not linear (Harder and Thomson, 1989). If visitation is infrequent, then higher per-visit pollen removal rates should reduce the amount of pollen that is never removed from the anthers and is effectively “wasted” (Harder and Thomson, 1989; Young and Stanton, 1990). However, the rate of male mating success may increase but at a decelerating rate with per-visit removal as a function of pollen loss (Harder and Thomson, 1989; Klinkhamer et al., 1994; Thomson et al., 2000). Thus, when visitation is frequent, a plant may increase male mating success by limiting the amount of pollen removed per-visit (Harder and Thomson, 1989; Klinkhamer et al., 1994). This effect may be magnified if pollen loss via grooming increases with pollen load size, as we may expect for pollen foraging visitors. We did not measure pollen removal in our study, and lupines characteristically employ dynamic pollen release as a function of visitation frequency (Harder and Thomson, 1989), so it is unclear what effect, if any, increased visit duration may have had on pollen removal in this system.

Second, we did not consider geitonogamous pollen transfer. Because of the positive relationship between visit duration and single-visit pollen deposition that we observed, increased visit duration may decrease male mating success by increasing pollen discounting through geitonogamy (de Jong et al., 1993). In general, individual plants with larger floral displays may export more pollen overall (Queller, 1997, 1983; but see: Broyles and Wyatt, 1990, 1995, 1997; Wilson et al., 1994). However, they should also suffer higher pollen discounting costs through geitonogamy, especially in self-incompatible species, whereas individuals with smaller displays may be less attractive and therefore struggle to export their pollen (Klinkhamer and de Jong, 1993; Klinkhamer et al., 1994). In this case, a novel nectar and pollen rewarding phenotype may reduce male mating success in larger plants by compounding pollen discounting. Conversely, if nectar rewards were able to reduce the threshold display size required to attract foragers, nectar rewards may increase pollen export, and potentially male mating success, in smaller plants. In our dataset, this would appear as a steeper positive slope of the display size $\times$ total visits or display size $\times$ flowers probed per visit curves for the nectar-added plants than the control plants, which we did not observe in our study (Supplemental Figure S2.1a and b). However, the existence and magnitude of such an effect may vary with population density, patch-level variation in display size, and the composition of the co-flowering community, and is worth considering in future work on intra-specific reward variation. Interestingly, Golubov et al. (1999) found that nectarless (but pollen-rewarding) morphs of honey mesquite (*Prosopis glandulosa*) attracted fewer pollinators than nectar and pollen rewarding morphs. As a result, nectarless morphs experienced slightly lower reproductive success through female function compared to nectar and pollen rewarding morphs but higher reproductive success through male function. This

suggests that alternative reward strategies may have very different implications for fitness through female versus male function.

Many plant species tolerate consumptive pollen loss, and offer pollen as a floral reward, while many others protect it from consumption through a variety of mechanisms, including provisioning pollen with defensive secondary compounds (e.g., Cane, 2018), placement in difficult to groom areas (“safe sites”) on pollinators’ bodies (e.g., Tong and Huang, 2018), and encasing pollen in pollinaria (Johnson and Edwards, 2000). Why should some lineages protect their pollen from consumption while others offer it as a reward? It is likely that lineages which use pollen as a reward are better able to balance the costs of pollen loss against the benefits of pollen transport than those that provide other rewards (or no reward). If resource and reproductive opportunity costs of consumptive pollen loss are balanced against effective and reliable pollination by pollen-foragers, then there may be nothing to gain from providing alternative rewards, such as nectar. In such cases, providing alternative rewards could represent an unnecessary resource cost (e.g., water and photosynthate), but provide no fitness benefit. While nectar is not always costly (Harder and Barrett, 1992) and its cost may be context-dependent (Rutter and Rausher, 2004), significant costs of nectar production have been demonstrated in several species (Southwick, 1984; Pyke, 1991; Ordano and Ornelas, 2005). For a species such as *L. argenteus* that primarily grows in xeric soils, arid climates, and at high elevation, the water that would be required to produce nectar could represent a substantial resource cost. Thus, for plants that are visited by pollen foragers, a strictly pollen-rewarding strategy may represent accepting a high opportunity-cost (lost pollen) while allowing plants to minimize other reward allocation costs without compromising any of the benefit provided by pollinator visitation.

Rewards serve at the foundation of resource-service mutualisms. The existence of such mutualisms relies on the ability of the partner that provides resources to balance the costs of those resources against the benefit they derive from interacting with the consumer (Bronstein, 2001). The evolutionary trajectories of reward strategies should be shaped by the unique combination of limitations and opportunities experienced by a lineage, and should, in-turn, define which species they can form and maintain mutualistic interactions with. Floral rewards are the primary ecological link between pollinator behavior and plant fitness (Parachnowitsch et al., 2018). However, we know little about the drivers of selection on floral rewards, the tradeoffs associated with alternative reward strategies, or the feedbacks between floral reward strategies and pollinator behaviors, reproduction, and evolution. Here, we found 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 outcome of the interactions between this species and its pollinators. Experimental and theoretical studies that explicitly examine the links between reward traits, mutualist behavior, and plant fitness are needed to help us understand the evolution of reward strategies (Parachnowitsch et al., 2018) and their significance to plant reproduction.

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DATA ACCESSIBILITY

The data used in this study will be achieved at and publicly available from Open Science Framework.

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Table 2.1. Means and standard errors of *Lupinus argenteus*

reproduction from plants with addition compared to control plants.

The nectar-added plants offered pollen and nectar rewards while control plants offered only pollen.

Response	Treatment	Mean	SE
Total seed production	Control	41.111	9.496
	Nectar addition	31.867	4.757
Seeds per fruit	Control	1.248	0.082
	Nectar addition	1.031	0.086
Aborted seeds per fruit	Control	0.812	0.128
	Nectar addition	0.855	0.064
Individual seed mass (mg)	Control	16.09	0.741
	Nectar addition	14.83	0.660

Table 2.2. Means and standard errors of *Lupinus argenteus*

reproduction from the pollen-limitation experiment (pollen

supplemented plants had pollen added to their stigmas whereas

control plants received no supplemental pollen and were open

pollinated).

Response	Treatment	Mean	SE
Proportion fruit set	Control	0.177	0.022
	Pollen supplementation	0.181	0.034
Total seed production	Control	55.364	8.651
	Pollen supplementation	38.000	6.608
Seeds per fruit	Control	1.068	0.094
	Pollen supplementation	1.019	0.109
Aborted seeds per fruit	Control	0.573	0.066
	Pollen supplementation	0.641	0.128
Individual seed mass (mg)	Control	17.855	0.854
	Pollen supplementation	16.869	1.197

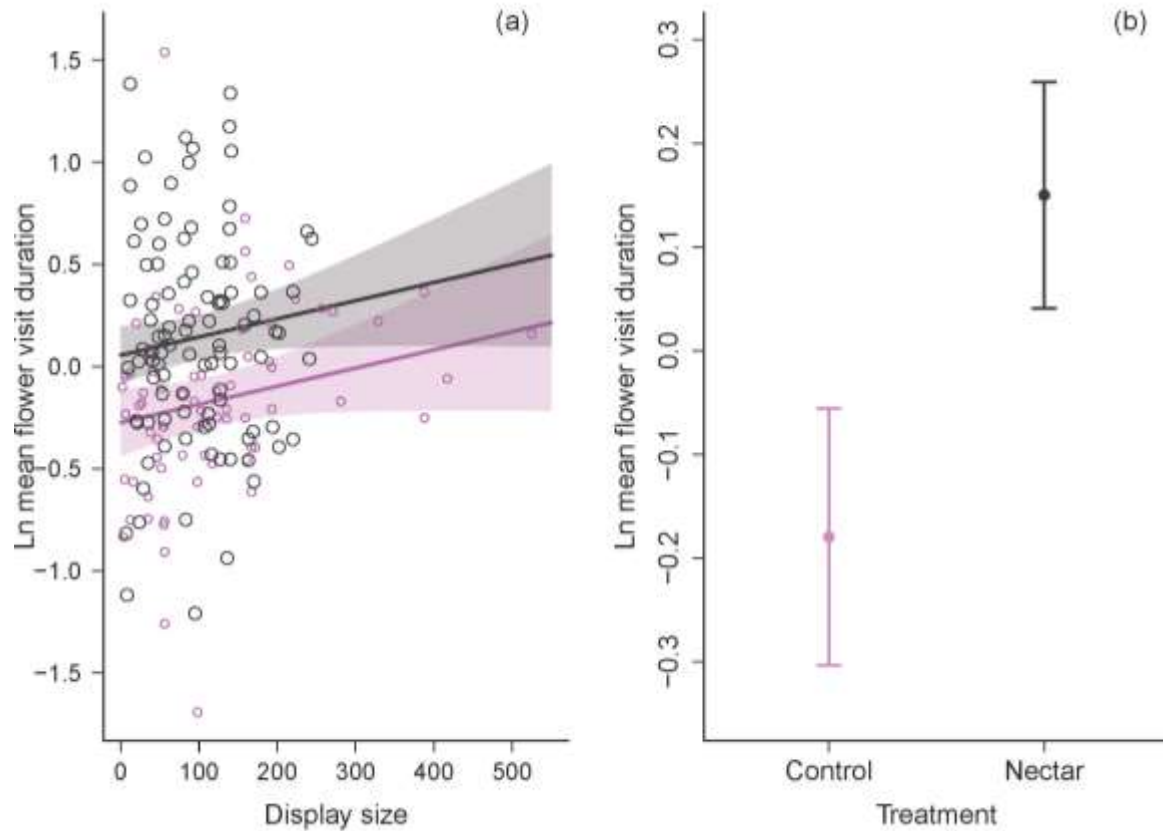


Figure 2.1. (a) Regression of the time (sec) that visitors spent per flower (natural log-transformed visit duration). The black line is the fit for the nectar-added plants (pollen + nectar), the violet line is the fit for control plants (pollen only). Large black circles are natural log-transformed data points for nectar-added plants, small violet circles are for control plants. Bands represent 95% CI; (b) Effects plot for LMM showing the main effect of nectar addition on the natural log of visit duration.

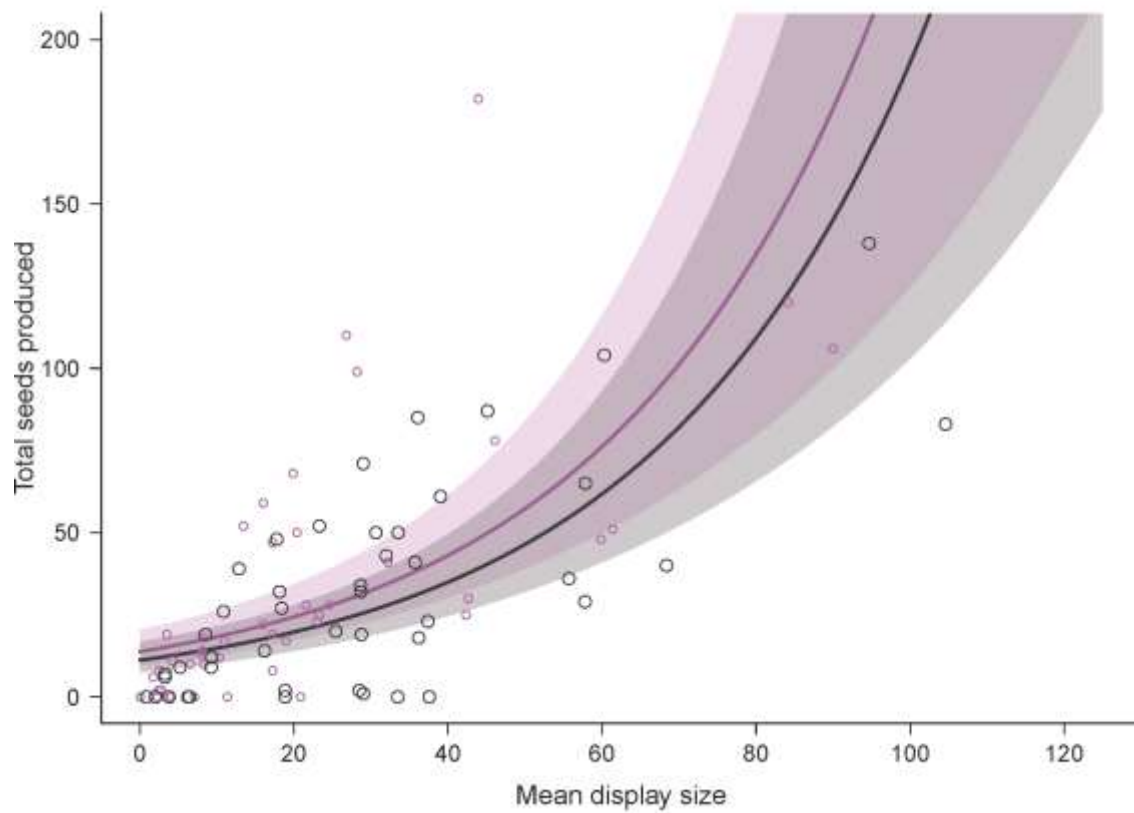


Figure 2.2. Total seeds produced per individual in the nectar addition experiment. The black line is the fitted curve for the nectar-added plants (pollen + nectar), and the violet line for control plants (pollen only). Large black circles are datapoints for nectar-added plants, and small violet circles for control plants. Bands represent 95% CI.

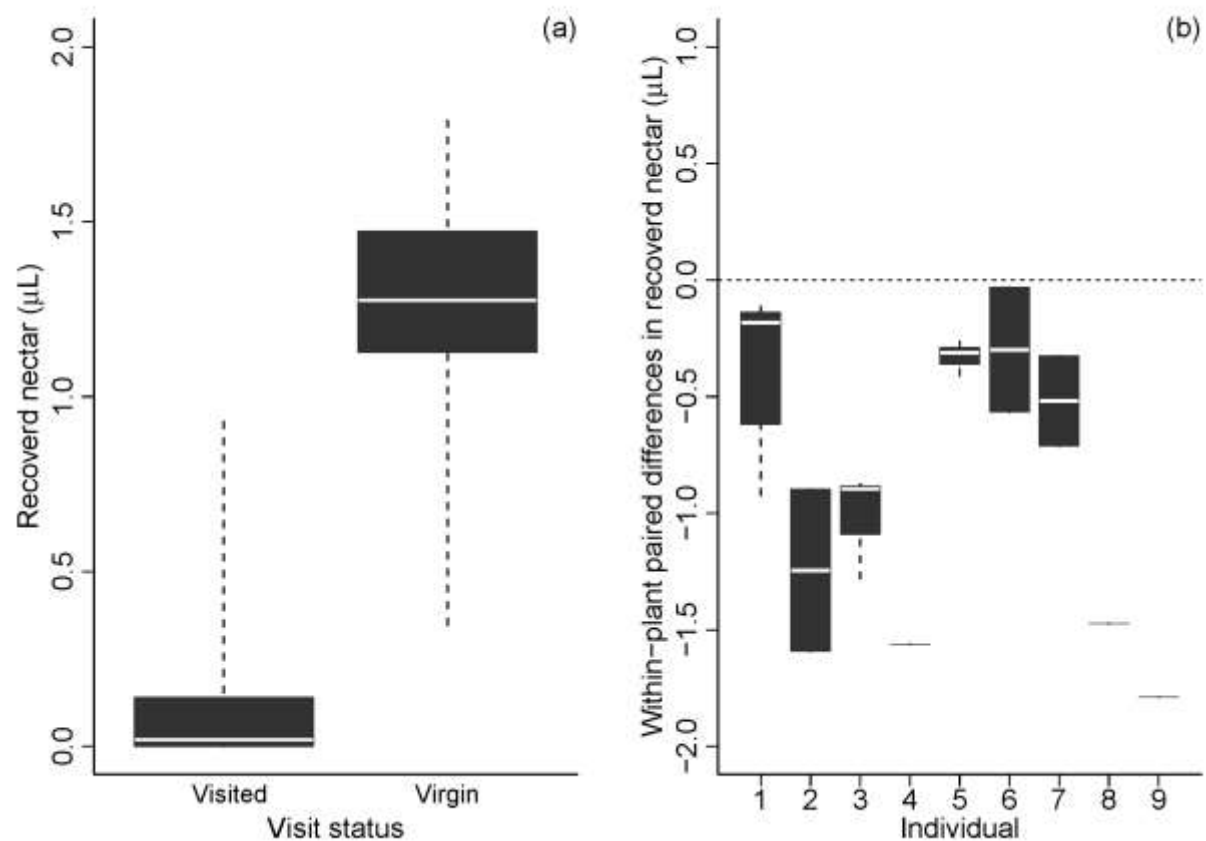


Figure 2.3. Absolute volumes (a) and differences in volume (b) of artificial nectar recovered from paired visited and virgin flowers within single stalks of *Lupinus argenteus* following nectar removal by bees. Visited flowers contained less artificial nectar than unvisited flowers. Bars are boxplots, 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.

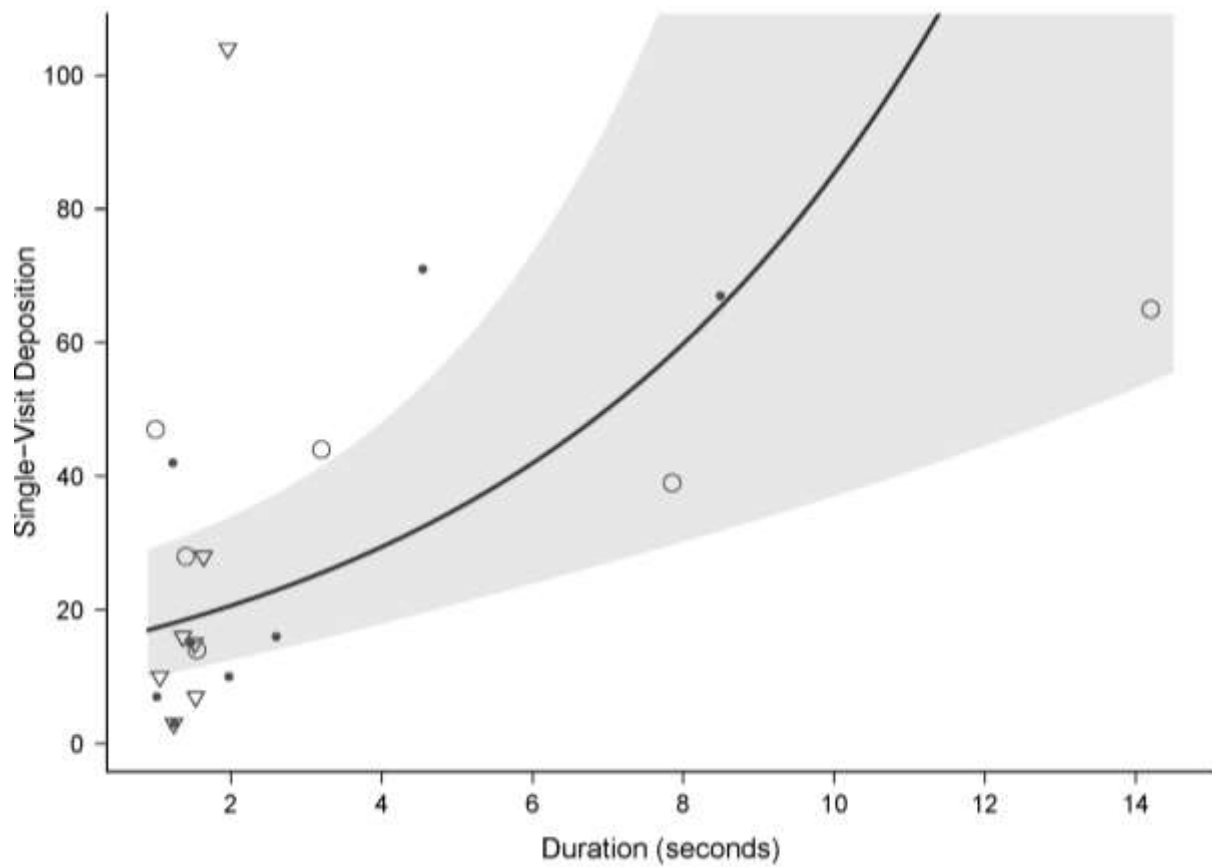


Figure 2.4. Single-visit pollen deposition increased with per-flower visit duration (sec). Dots are raw data points, with treatments differentiated by symbol, where 0μL = triangles, 2μL = small filled circles, 4μL = large hollow circles. The band represents 95% CI.

CHAPTER 3: Pollinator Preference and Grooming Place Conflicting Constraints on Pollen Donation in Pollen-Rewarding Plants.

Jacob M. Heiling, Rebecca E. Irwin, and William F. Morris

ABSTRACT

Some plant species offer pollen as their sole reward to pollinators, creating an inherent conflict between the production of pollen as reward vs. gamete. How do flowering plants presenting only pollen as a reward minimize the effects of consumptive pollen loss on male plant reproduction? To address this question, we modeled the effects of pollen production and the division of pollen among visitors on pollen donation, accounting for pollen loss and reward size preference by pollinators. We found that pollen-rewarding plants can maximize male reproductive success by increasing pollen production and dividing pollen among many large “packages” (units of pollen available per flower per visit). However, there are limits to the benefits of larger package sizes in pollen rewarding plants. When consumptive pollen loss is very high, the benefits to male function associated with producing larger packages fails, possibly resulting in selection for reduced package sizes. Our results suggest that conflicting constraints on pollen donation through pollinator preference and grooming, together, explain the large investments in pollen observed in many pollen-rewarding plants. Further these constraints are likely to limit reproductive success through male function in pollen-rewarding plants.

INTRODUCTION

In outcrossing plants, male mating success depends on the dispersal of pollen from one individual (pollen donor) to the stigmas of another (pollen recipient). Pollen dispersal is not an efficient process for most plant species: the vast majority of the pollen that is removed from a donor (>95% in some cases) never reaches a recipient stigma (Harder and Thomson, 1989; Lertzman and Gass, 1983; Minnaar et al., 2018). For animal-pollinated plants, some of this pollen loss is passive, with pollen falling from the flower or the visitor (Thomson, 1986), and some can be attributed to pollen placement on heterospecific or genetically incompatible flowers (Inouye et al., 1994; Morales and Traveset, 2008). However, the majority of this loss can be attributed to the grooming behaviors of pollinators, which can represent simple cleaning behavior or serve to consolidate and store pollen for transport during pollen foraging (Thomson, 1986; Harder and Thomson, 1989; Koch et al., 2017; Minnaar et al., 2018).

Typically, a flower should not make all of its pollen available to its visitors at once. This is because the rate of pollen donation to compatible stigmas may slow as the number of grains available per visit increases due to increasing proportional pollen loss at the flower, among flowers within a plant, and loss due to consumption or body-cleaning by the pollinator (Thomson et al., 2000; Harder and Thomson, 1989; Lloyd, 1984). If pollinator availability were unlimited, then depositing a single grain on each visitor would give the highest likelihood that a given grain would reach a receptive stigma (Harder and Thomson, 1989; Klinkhamer and de Jong, 1993). However, visitation is not unlimited and floral visitors are not perfect pollen vectors. They lose many grains, both passively and due to grooming; thus, plants often must deposit considerably more pollen on each visitor than the ideal of a single grain (Harder and Thomson, 1989; Klinkhamer and de Jong, 1993).

Pollen donation is not only limited by loss following removal from anthers, but also by the likelihood that it is removed from the potential sire's anthers in the first place. Strictly pollen-rewarding plants are those that provide pollen as their only floral reward to pollinators. Nearly 20,000 species of insect-pollinated angiosperms use pollen as their sole floral reward (Willmer, 2011). When pollen is the sole reward offered by a plant, the probability of removal should be influenced by an interaction between pollen availability and the reward-size preferences of pollen-foraging pollinators. This means that, for pollen-rewarding species, the challenges to pollen donation presented by pollen loss may be compounded by reward-size preferences of pollinators, which should favor larger pollen rewards. Thus, the minimum number of pollen grains that a pollen-rewarding plant must offer to a pollinator may far exceed the number that would be optimal for minimizing pollen loss. Existing theory does not explicitly consider how selection should operate on pollen investment and presentation when pollen is not only a gamete in need of transport, but also a pollinator reward. Darwin (1859) argued that loss of pollen to consumption should select for increased pollen production to maintain male plant fitness. This hypothesis is intuitively satisfying. Indeed, there is some empirical evidence that strictly pollen-rewarding plants produce more pollen than their nectar-rewarding relatives (Simpson and Neff, 1983; Golubov et al., 1999; Etcheverry et al., 2012). However, the effect of pollen production on realized pollen donation should be shaped by the interaction between reward size preference and pollen loss, and the effect of this interaction on pollen presentation. The interactions between these factors, and their implications for allocation and presentation dynamics in pollen-rewarding plants have yet to be explored in the theoretical literature.

Here, we present an analytical model that incorporates the dual constraints of reward size preference by pollinators and pollen loss through grooming. We use this model to explore the

effects of pollen production and packaging (the amount of pollen available in a flower in a single pollinator visit) on pollen donation in pollen-rewarding plants. We started with a pollen donation and loss model originally developed by Harder and Thomson (1986) and modified it to account for pollen-forager reward size preferences. We used this model to address the following questions: (1) How do grooming and reward size preference interact to affect pollen donation? (2) How does pollen production affect pollen donation given the constraints of grooming and reward size preference? And (3) does making more pollen compensate for consumptive pollen loss when foragers show reward size preference?

POLLEN DONATION MODEL

Pollen grain donation with pollen loss

Many animal pollinated plants exhibit mechanisms to control the release of pollen over a period of time (Lloyd & Yates, 1982; Thomson et al., 2000). Plants can employ a large array of architectural and phenological mechanisms to control pollen release both within individual flowers, and across the entire floral display (Harder and Thomson, 1989; Lloyd and Yates, 1982). We use the term *pollen packaging* to refer to the amount of pollen available in a flower to a visitor during a single visit. In this study, we do not differentiate between pollen packaging and pollen dispensing (Harder & Thomson, 1989; Li et al., 2014), because the questions that we address in this study do not focus on specific mechanisms by which pollen release is controlled. Further, because we are limiting our focus to processes affecting pollen fate at a per-flower level, we do not consider how pollen is divided among flowers on multi-flowered plants, variable pollen dispensing schedules, or geitonogamous pollen transfer. The roles of these factors on

pollen donation are described elsewhere in the pollen presentation literature (e.g., Harder and Thomson, 1989; Harder and Wilson, 1994; Castellanos et al., 2005; Thomson, 2006).

Few of the pollen grains that leave a flower will ever reach a conspecific stigma, with estimates as low as 3-6 % for some plant species (Lertzman and Gass, 1983; Harder and Thomson, 1989; Minnaar et al., 2018). The avenues of pollen loss can be divided into two main pathways: grooming and passive loss (Harder and Thomson, 1989). *Pollen grooming* describes the processes by which pollinators remove pollen from placement sites on their bodies to discard it, consume it, or store it for subsequent consumption by mates or colony members (Boggs & Gilbert, 1979; Inouye et al., 1994; Thomson, 1986). *Passive loss* refers to pollen that is lost through processes other than grooming and consumption by pollinators or deposition on stigmas, such as pollen that falls from the flower during floral manipulation or from the pollinator's body in flight (Inouye et al., 1994; Thomson, 1986). Pollen lost through either avenue is pollen that will never reach a conspecific stigma and represents a loss to the donor's total siring success (Harder & Thomson, 1989; Minnaar et al., 2018; Thomson et al., 2000).

We used an equation (Eq. 1) from Harder and Thomson (1989) to represent the number of grains donated, D , from a package of size R , conditional on its removal by a visitor and accounting for passive loss and grooming, which are governed by the attenuation coefficients a and g , respectively (Table 3.1):

$$D = a R^g(1)$$

with $0 \leq a \leq 1$ and $0 < g \leq 1$ (Table 3.1). Here, the case of $a = 0$ represents passive loss of all pollen removed during a visit, and the amount of pollen that is passively lost decreases as a approaches 1. If $g = 1$, the fraction deposited is independent of the amount removed, but if $g < 1$, the fraction deposited declines with the amount removed, as would be expected if the visitor is

more likely to groom and/or to groom more thoroughly after picking up a larger amount of pollen (Castellanos et al., 2005; Harder, 1990b). If $a > 0$ and $0 < g < 1$, D is an increasing but decelerating function of R (Fig. 3.1). The grooming attenuation coefficient g can also range from 0 to 1, with $g = 0$ representing the loss of all pollen removed from a flower to grooming and passive loss (when $a > 0$) (Table 3.1). The amount of pollen that is lost to grooming decreases as g approaches 1 (Fig. 3.1). When grooming intensity is low (larger values of g), pollen packages display high donation efficiency, which we define here as the proportion of grains donated per package.

We were not explicitly interested in the effects of variation in passive loss in this study, and so we used a single value of a for all calculations. Harder and Thomson (1989) found that a mean of 14% of the pollen available in nectar-rewarding *Erythronium americanum* flowers was lost passively during manipulation of flowers by nectar-foraging *Bombus* (*B. ternarius* and *B. terricola*) queens and *Apis mellifera* workers, while Rademaker et al. (1997) found that approx. 50% of the pollen removed during single visits to nectar-rewarding *Echium vulgare* flowers failed to adhere to nectar foraging *Bombus terrestris* workers. These values translate to passive loss coefficients of $a = 0.86$ and 0.5 , respectively. Because pollen-rewarding plants often exhibit some degree of specialized pollen placement on or presentation to pollinators (Dunn, 1956; Harder, 1990a; Vallejo-Marín et al., 2009), we held passive loss constant at $a = 0.9$ (Table 3.1). This value represents lower passive loss (10%) than that observed by Harder and Thomson (1989) and Rademaker et al. (1997) in the nectar-rewarding species they studied which exhibit no specialized pollen placement. Actual passive loss values may be considerably lower in plants that exhibit very specialized placement and/or high production of pollenkitt (e.g., *Lupinus* spp.) or higher in those with large open and unspecialized corollas (e.g., *Papaver* spp.).

Incorporating pollen reward size preference

To account for the effect of reward size preferences on pollen removal, we used a Michaelis–Menten equation to scale the probability of removal p of a given package of size R to the degree of a pollinators' reward-size preference y (Fig. 3.2) (Table 3.2):

$$p = \frac{R^y}{H^y + R^y} \quad (2)$$

The case $y = 0$ represents visitors that are completely indifferent with respect to the size of pollen rewards. Reward size preference, i.e., the degree of discrimination with respect to reward size, increases as y increases. As reward size preference increases, package size must also increase to maintain the same removal probability (Fig. 3.2). Here, H is similar to the Michaelis half saturation constant and, when visitors discriminate ($y > 0$), is equal to the package size, i.e., the number of pollen grains, at which the probability of removal equals 0.5 (Table 3.1). Thus, the effects of preference on removal are determined by both y and H . Setting $H = \frac{A}{2}$ in Eq. 2 allows the rate of increase of the probability of removal to decelerate rapidly after some threshold package size. We should predict such a deceleration when visitor preferences saturate due to cognitive, physiological, or handling-time limitations and when visitation is limited (Fig. 3.2). In Figure 3.2, all three curves intersect at 5,000 grains per package (i.e., $1/2$ of A , which is 10,000 in this case). The point of intersection could shift up or down on the x-axis for alternative values of H without qualitatively changing these results.

Optimizing per-package and per-flower pollen donation

The product of Eq. 1 and Eq. 2 gives the total pollen donation expected per package f :

$$f = pD \quad (3)$$

(Table 3.2). If we make the simplifying assumption that packages produced by a single flower are of a constant size, R , then the number of packages that each flower produces in its lifetime n is (Table 3.2):

$$n = \frac{A}{R} (4)$$

Thus, the total expected pollen donation per-flower F is the product of the number of packages (n) and the total pollen donation expected per package (f) (Table 3.2):

$$F = nf (5)$$

By holding A , H , y , a , and g constant, we searched over the range of R (in 100 grain intervals from 100 to A) for the package size that maximizes total per-flower pollen donation (F), which represents the optimal package size from the perspective of siring success. By plotting the optimum package size against the value of y , we determined how discrimination and grooming jointly constrain optimal package size. And by examining the value of F (eq. 5) at the optimum R , we examined how discrimination affects male success at optimal packaging (Figure 3.3A&B).

Total per-flower pollen production

By varying the total pollen produced per-flower (A), we can examine how pollen donation changes over the range of possible package sizes. Whereas y governs the effect of package size on removal probability (p) independent of total per flower pollen production, H ties removal probability to total pollen production. Because H increases with A (Table 3.2), the effect of increased pollen production on removal probability is to increase the package size required to achieve a given removal probability (Fig. 3.4D&F). This relationship implies competition for visitation, and thus removal, among individuals expressing variable package sizes for a fixed level of production. In our model, we examined the effect of production on donation using three

levels of per-flower pollen production (10,000; 50,000; and 100,000 grains/flower). These values are based on those of *Lupinus sericeus* (Harder, 1990a), *Desmodium uncinatum* (Etcheverry et al., 2012), and *Solanum chimborazense* (Mione and Anderson, 1992), all of which are strictly pollen rewarding. To evaluate how the interaction between forager preferences and pollen loss to grooming may affect pollen donation, we investigated how package size optima change across the ranges of preference (y) and the grooming coefficient (g) while holding total pollen production (A) constant.

Assumptions

For simplicity, our model assumes that pollinator availability is only limited by discrimination due to package size, and that package size does not change in response to visitation frequency. These assumptions allow for a simple model with which we can address our questions, avoiding limiting our results to the context of a specific system. However, this model can serve as a basis to build more system-specific models, or to account for other pollen presentation characteristics or dispersal dynamics by relaxing these assumptions.

RESULTS

(1) How do grooming and reward size preference interact to affect pollen donation?

Grooming and reward size preference interact to limit pollen donation. While grooming limits donation through pollen loss, reward size preferences limit removal and shift package size optima to higher values than would be obtained in the absence of reward size preferences.

By definition, the probability of pollen removal does not change across the range of possible package sizes when foragers are indiscriminate with respect to reward size (low values of y , solid curve, Fig. 3.2). However, as package size preference increases (intermediate and high y values, dashed and dotted curves, Fig. 3.2), foragers are less likely to remove smaller packages but more likely to remove larger packages.

Figure 3A shows the optimum package size for three values spanning the range of g plotted over the range of pollinator discrimination, y . Optimal package size increases with reward size preference, but the effect is severely dampened by increasing grooming intensity (decreasing values of g). Likewise, Figure 3B shows optimal package size curves for three values spanning the range of y plotted against the range of g . Optimal package size decreases as grooming intensity increases; however, it does so more slowly as reward size preferences increase (higher values of y).

(2) How does pollen production affect pollen donation, given the constraints of grooming and reward size preference?

Under the constraints imposed by grooming and reward size preference, increasing pollen production can increase total donation but does not improve donation efficiency. When grooming is intermediate or high, as we would expect if visitors are foraging for pollen (Harder, 1990b), proportional pollen donation decreases (Fig. 3.4A-C) as total production increases. Thus, increasing total pollen production (per-flower) decreases pollen donation efficiency. However, the total number of grains donated does increase with total pollen production (Fig. 3.4D-F), at least over intermediate levels of grooming intensity.

(3) Does making more pollen compensate for consumptive pollen loss when foragers show reward size preference?

The primary benefit of increasing pollen production may be that it allows a plant to make many packages, helping mitigate the effects of pollen loss on total male mating success, while also making those packages large enough to attract pollinators. Although donation efficiency is lower when more pollen is made, when pollen package size is optimized, maximum fitness is obtained through higher pollen production, regardless of grooming intensity and reward size preference (Fig. 3.5). However, the difference in maximum male fitness returns between pollen production levels diminishes at very high grooming intensities (Fig. 3.5). This implies that any benefit of increased pollen production could be diminished or lost (Fig. 3.5C) when grooming intensity is sufficiently high. When foragers show very low reward size preference ($\gamma = 0.01$), optimal package size is constant at the minimum size allowed by our model (100 grains) across the range of g regardless of the level of total pollen production (Fig. 3.6A). As forager reward size preference increases, increasing pollen production yields higher optimal package sizes when grooming intensity is low to intermediate (Fig. 3.6B&C). However, optimal package size converges across all production levels and eventually collapses to the minimum for our model (100 grains) as grooming intensity continues to increase. This collapse occurs closer to $g = 0$ as forager preference increases (Fig. 3.6B&C).

DISCUSSION

Our modeling results illustrate that, for pollen rewarding plants, pollen loss and pollinator preferences should place conflicting selection on pollen package size. Our results suggest that these drivers of selection may result in the production of large pollen packages, favoring removal

over minimizing loss. Pollen rewarding plants may be able to maximize pollen donation in the face of the conflicting demands imposed by grooming and reward size preference by producing large quantities of pollen and dividing it among many large packages. Finally, since the value of producing large packages may collapse when preferences are very strong and foragers groom very efficiently, these same forces may interact to limit the degree to which forager preferences can drive pollen reward size evolution. Understanding the interaction between pollinator preference and pollen loss represents a gap in the pollen presentation literature that this research fills. Further, our results provide a mechanistic explanation for higher pollen production in pollen rewarding plants, while indicating that increased pollen production alone is not a sufficient strategy for coping with pollen consumption.

With two opposing forces defining optimal package size, dividing total pollen production among packages of some intermediate size may be a simple, if not completely effective, strategy for coping with these limitations. However, if competition for pollinators is strong, or if grooming intensity increases along with package size, this may result in very low overall male mating success. Intraspecific competition for pollinators could also reduce female success in plants that produce smaller packages. As we have shown here, producing more pollen and dividing it among many large packages may present a compromise, allowing plants to maximize both removal and donation under the limits imposed by preference and pollen loss.

Beyond some grooming intensity threshold, the benefit of competing for removal may fail, with optimal package size collapsing and approaching 1 regardless of total pollen production level (Fig. 3.6B&C). At this point, very high pollen loss effectively eclipses the benefit of competing for removal (far right-hand side of Fig. 3.6B&C). Likewise, selection on package size may be driven by the need to minimize grooming and consumptive loss, but not by reward size

preference, and much of the benefit of higher pollen production and large package sizes may vanish. Thus, if foragers become too picky and groom too well, selection on package size may in pollen-rewarding species may mirror that in non-pollen rewarding species, possibly resulting in smaller package sizes.

Bees constitute perhaps the most important pollen-foraging pollinators in most terrestrial plant communities (Michener, 2000; Thorp, 2000; Heinrich, 2004; Willmer, 2011) due to their dependence on pollen as a protein source (Simpson and Neff, 1983) coupled with their abundance, wide occurrence, morphological and behavioral characteristics, broad environmental tolerances, and high foraging efficiency. Bees are known to assess pollen quality and quantity while foraging (Ruedenauer et al., 2015; Muth et al., 2016; Nicholls and Hempel de Ibarra, 2017), and the economics of pollen foraging with respect to reward assessment and movement decisions may mirror those observed among nectar foragers (Rasheed and Harder, 1997a, 1997b). Thus, traits of pollen-rewarding species may be disproportionately influenced by their interactions with bees relative to other pollen foragers. Even when foraging for nectar, bees efficiently remove pollen from their bodies (Harder and Thomson, 1989; Thomson, 1986; Thomson and Plowright, 1980). Grooming efficiency should be at least as high when bees are pollen-foraging, though this remains unknown and should be the subject of future work. Likewise, bees are known to discriminate on the basis of reward size and quality when pollen-foraging (Harder, 1990b; Muth et al., 2016; Rasheed and Harder, 1997b; Ruedenauer et al., 2015), as they are well known to do when nectar-foraging (Ackerman, Rodriguez-Robles, and Melendez, 1994; Cresswell and Galen, 1991; Goulson, 2010). Thus, when considering male fitness returns for pollen-rewarding nectarless plants, true values of g should be rather low and reward size discrimination should be expected to exert a significant influence on pollinator

behavior. If so, then pollen foraging bees may greatly constrain male fitness for pollen-rewarding plants.

Being a pollen-rewarding plant inherently imposes a high opportunity cost on male mating success through gamete loss. As we demonstrate, the need to attract pollinators may further compound this mating cost by driving increases in pollen package sizes. But, using pollen as the sole floral reward likely represents the ancestral condition in angiosperms (Simpson and Neff, 1983), and many angiosperm species have maintained or secondarily derived this reward strategy, with strictly pollen-rewarding genera, sub-genera, and species scattered throughout lineages containing many nectar rewarding taxa (e.g., *most Acacia spp.*, Stone et al., 2003; some *Desmodium spp.*, Etcheverry et al., 2012; *Lupinus spp.*, Dunn, 1956; *Ononis spp.*, Vivarelli et al., 2011; *Papaver spp.*, Simpson and Neff, 1983; some *Pedicularis spp.*, Macior, 1970; *Piper spp.*, de Figueiredo and Sazima, 2000; *Solanum spp.*, Simpson and Neff, 1983; Kessler and Halitschke, 2009). Are there any counterbalancing benefits for these plants? There are at least two ways that the benefits of using pollen as a sole floral reward may benefit plants, both relating to the nature of the plant—pollen-forager interaction. First, if pollen-foraging bees visit a nectar-producing species primarily for its pollen, then a loss of nectar production may conserve resources that would otherwise represent an unnecessary expense. Such a mechanism would only apply to species for whom nectar production is particularly costly relative to the resources required to produce pollen (Pyke, 1991; Rutter and Rausher, 2004; Ordano and Ornelas, 2005; but see: Harder and Barrett, 1992). Although pollen is nitrogen rich relative to most nectar (Simpson and Neff, 1983; Roulston and Cane, 2000), pollen rewards could represent a lower cost alternative to nectar rewards for plant species that are more limited by water availability than nitrogen, such as nitrogen fixers in arid or semi-arid environments (e.g., *Lupinus* and *Acacia*

species). Further, for such species in communities that include nectar-rewarding co-flowering heterospecifics, offering substantial pollen rewards may be a cost-effective strategy in interspecific competition for pollinator visitation. Indeed, Tong et al. (2018) argued that differentiation between nectar and pollen-rewarding strategies between pairs of co-occurring *Pedicularis* species may facilitate co-existence. Such explanations could be assessed through a combination of comparative phylogenetic studies that account for variation in floral reward strategies and the resources dynamics across species and community-level studies of intraspecific competition for pollinators that take reward strategies into account.

Second, there is empirical evidence that suggests foragers may move within and between inflorescences differently depending on whether they are foraging for pollen or for nectar (Galen and Plowright, 1985; Haynes and Mesler, 1984). For example, pollen foragers may make fewer within-plant movements than nectar foragers (Haynes and Mesler, 1984). If such differences in movement patterns are common, then a pollen-rewarding strategy may be associated with lower geitonogamous pollen movement. Such decreased geitonogamous loss could provide a substantial benefit in the face of visitation by pollen-foragers and may even, from the perspective of male function, balance or outweigh the cost of high pollen loss through grooming.

A non-adaptive explanation for transitions to a strictly pollen-rewarding strategy is that reward redundancy in species that provide nectar and pollen rewards may mean that maintenance of pollinator visitation may be robust to the loss of one reward. Thus, mutations inducing the loss of nectar production may not be selected against.

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DATA ACCESIBILITY

R scripts (R Core Team, 2018) we be made publicly available through the Open Science Framework.

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Table 3.1. Parameter meanings, ranges, and values used.

Parameter	Meaning	Range or values
A	Total pollen grains produced per flower	10,000; 50,000; and 100,000
R	Pollen package size, number of grains released/flower/visit	100 up to A
a	Attenuation coefficient for passive loss	0 – 1 constant at $a = 0.9$
g	Attenuation coefficient for grooming/consumptive loss	0 – 1
y	Reward size preference	0 – 1
H	Half-saturation constant for removal probability	$A / 2$

Table 3.2. Calculated values, meanings, and equations.

Calculated values	Meaning	Equation	Number
D	Total number of pollen grains donated per package (Harder and Thomson 1989)	$D = aR^g$	Eq. 1
p	Probability of package removal	$p = R^y / H^y + R^y$	Eq. 2
f	Total pollen donation per-package	$f = pD$	Eq. 3
n	Number of packages per-flower	$n = A / R$	Eq. 4
F	Total pollen donation per-flower	$F = nf$	Eq. 5

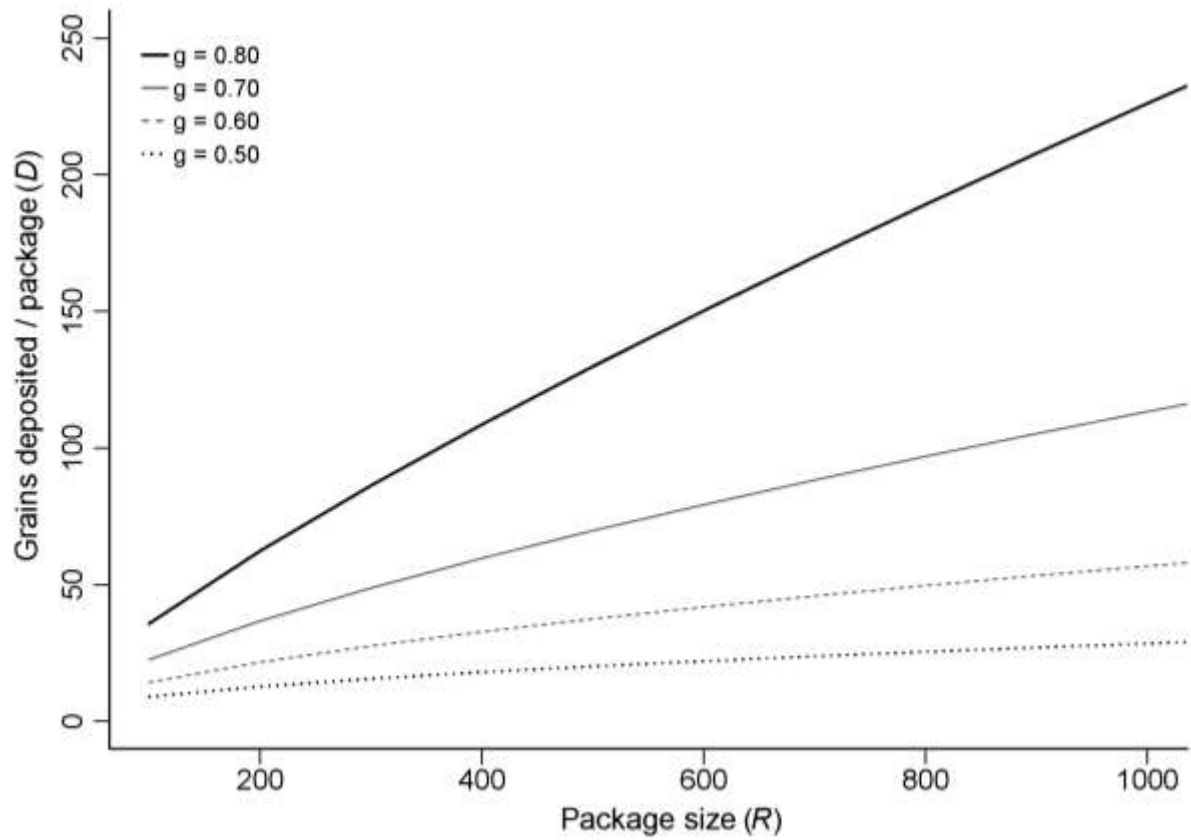


Figure 3.1 The effect of grooming on pollen donation: The number of grains donated per package (D) plotted against package size (R) according to Eq. (1) for four values of the grooming attenuation coefficient (g). The per-package donation fraction decreases as grooming intensity increases (decreasing values of g). $A = 10,000$; $\alpha = 0.9$.

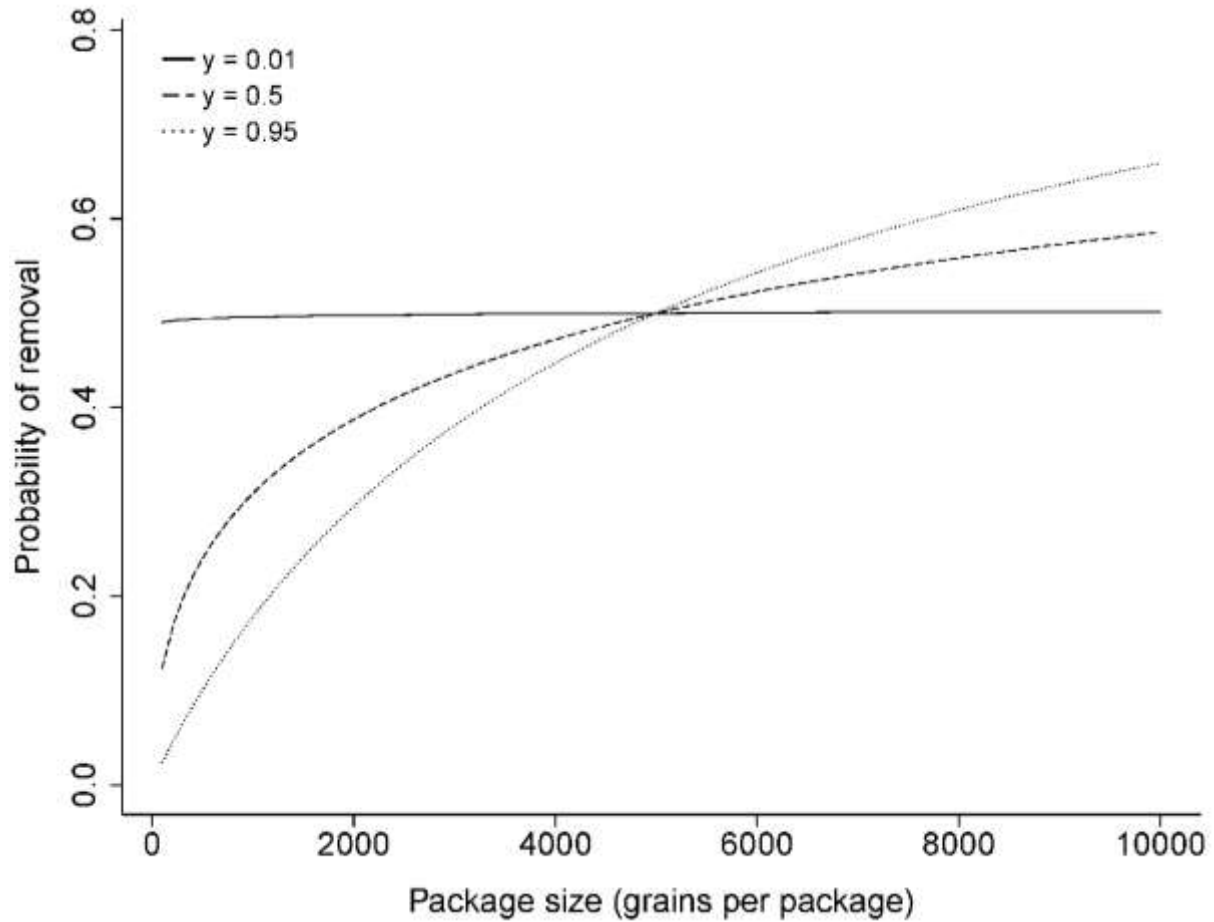


Figure 3.2 The effect of visitor preference on the probability of pollen removal: As pollen package size (i.e., reward size) (R) increases, the probability of removal increases across all levels of reward size preference (y). However, when reward size preference is low ($y = 0.01$ here), the effect of package size on removal probability is weak. When foragers show higher preference for reward size ($y = 0.5$ and 0.95), the probability of removal for packages below some threshold value decreases, while the probability of removal above that value increases relative to more permissive foragers. $A = 10,000$; $H = 5,000$; $a = 0.9$.

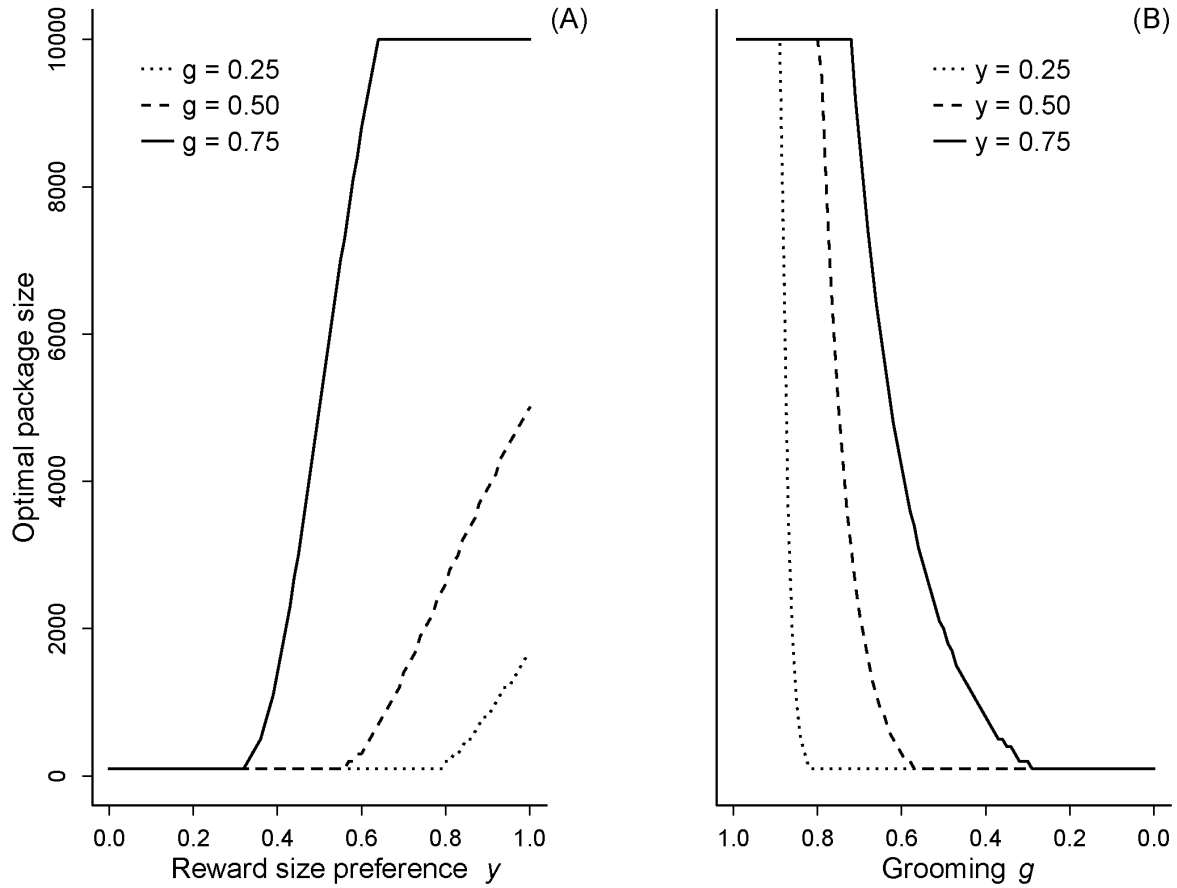


Figure 3.3 Panel A: Optimal package size (package size at which pollen donation is maximized) curves for three values of the grooming attenuation coefficient (g) plotted across the range of reward size preference (y). Panel B: Optimal package size curves for three levels of reward size preference (y) plotted across the range of the grooming attenuation coefficient (g). Low values of y correspond to foragers showing little preference for pollen reward (i.e., package) size; preference increases with y . Large values of g correspond to low values of active pollen loss (through grooming and consumption); loss to grooming increases as the value of g decreases (thus, the x-axis in panel B runs from 1-0). $A = 10,000$; $H = 5,000$; $a = 0.9$, both panels.

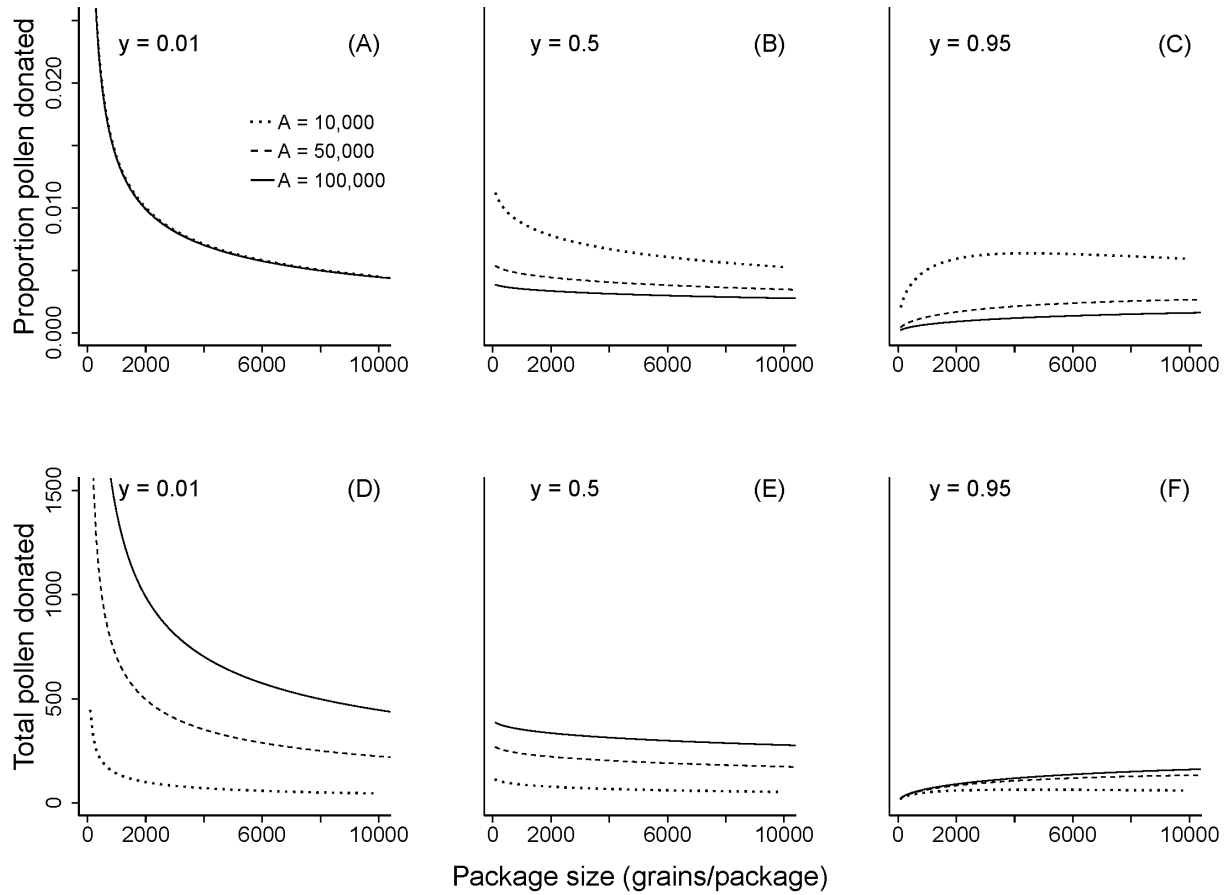


Figure 3.4 The effect of total pollen production (per-flower) and package size on: the proportion of pollen donated (panels A-C) and the total number of grains donated (panels D-F). Pollinator reward size preference increases from left to right. When grooming is intermediate (here: $g = 0.5$), pollen donation efficiency decreases (panels A-C) as total production increases. Thus, increasing total pollen production (per-flower) does not increase the proportion of pollen donated (panels A-C), but it does increase the total number of grains donated (panels D-F). When reward size preference is low (A&D, B&E) donation decreases as package size increases.

$$H = \frac{A}{2}; g = 0.5; a = 0.9, \text{ all panels.}$$

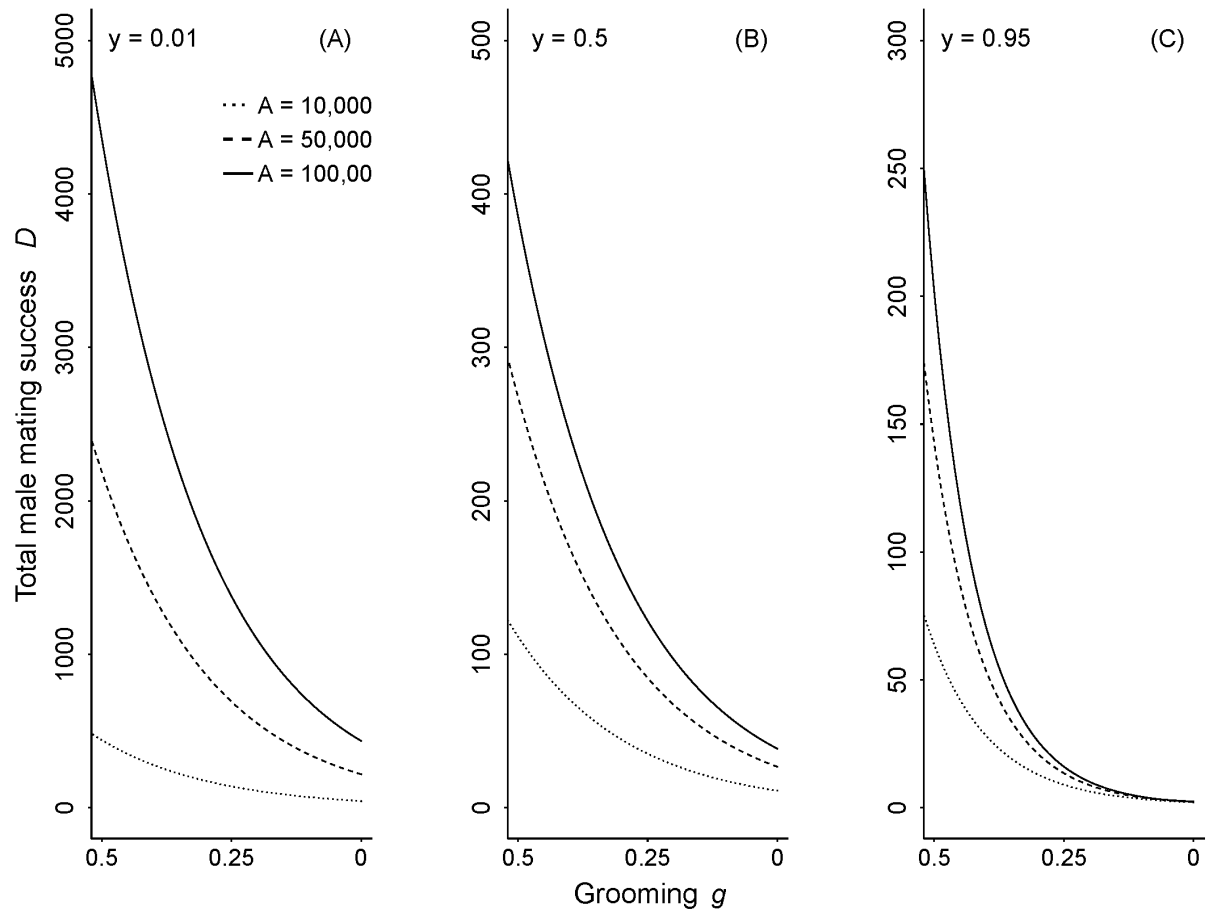


Figure 3.5 The effect of total pollen production (A) on total male mating success (pollen donation, D) at optimal package sizes under moderate to intense grooming ($g = 0.5-0$) for three levels of reward size preference. $H = \frac{A}{2}$; $a = 0.9$.

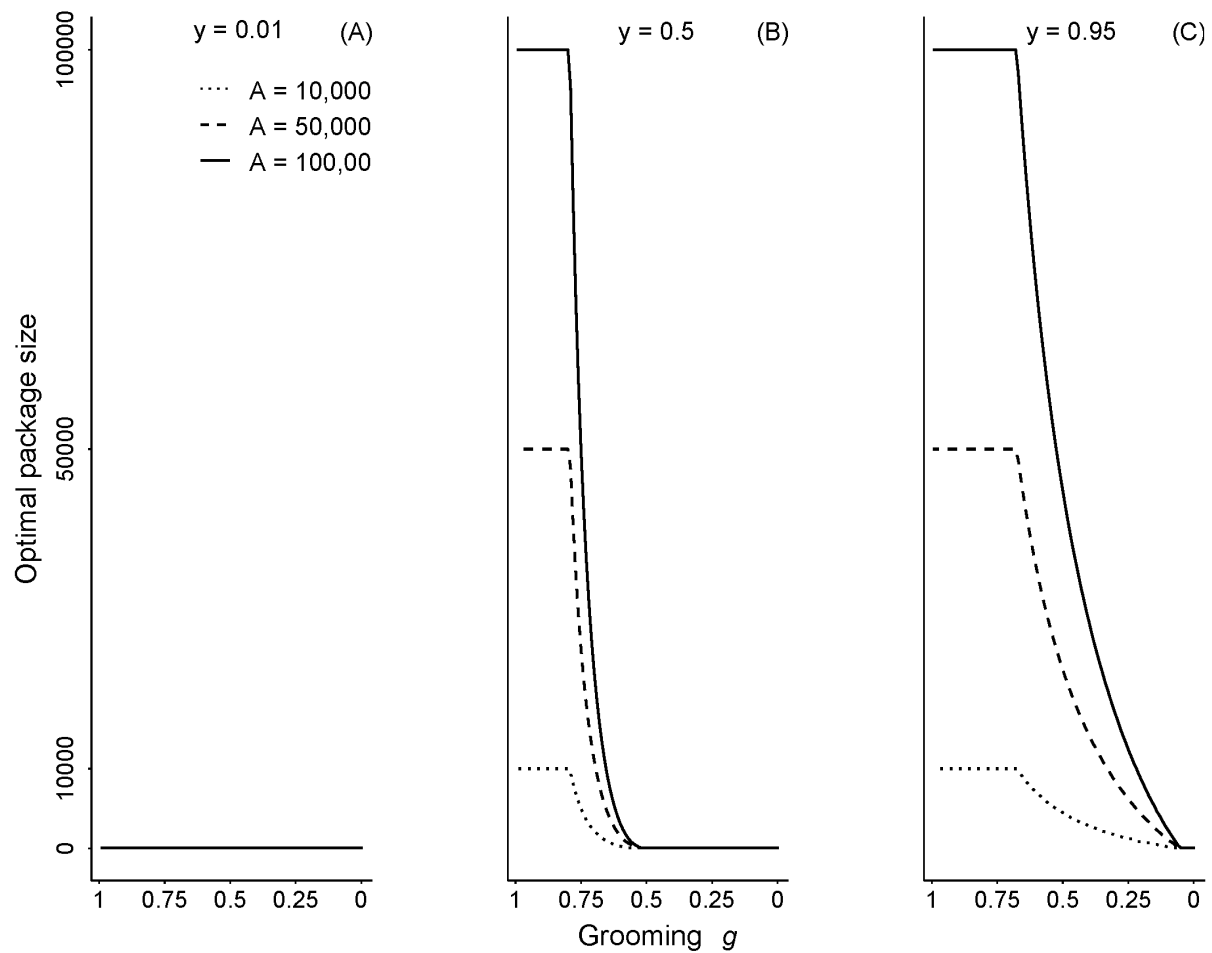


Figure 3.6 The effect of total pollen production (A) on optimal package size (package size at which pollen donation is maximized) across the range of the grooming attenuation coefficient ($g = 1-0$) for three levels of reward size preference, increasing from left to right. $H = \frac{A}{2}$; $a = 0.9$.

CHAPTER 4: Attract or defend? Pollen and Vegetative Secondary Chemistry of Three

Pollen-Rewarding Lupines

Jacob M. Heiling, Daniel Cook, Stephen T. Lee, and Rebecca E. Irwin

ABSTRACT

PREMISE OF THE STUDY: Optimal Defense Theory predicts that selection should drive plants to disproportionally allocate resources for herbivore defense to tissues with high fitness values. As pollen's primary role is the transport of gametes, plants may be expected to defend it from herbivory. However, for many animal-pollinated plants, pollen serves a secondary role as a pollinator reward. This may present a conflict between selection to defend pollen from herbivores and selection to reward pollinators. Here, we investigate whether pollen secondary chemistry in three pollen-rewarding *Lupinus* species better reflects the need to defend pollen or reward pollinators.

METHODS: *Lupinus* (Fabaceae) species are nectarless, pollen-rewarding, and produce defensive quinolizidine and/or piperidine alkaloids throughout their tissues. We used gas chromatography to identify and quantitate the alkaloids in four above-ground tissues (pollen, flower, leaf, stem) of three western North American lupines: *L. argenteus*, *L. bakeri*, and *L. sulphureus*, and compared alkaloid concentrations and composition among tissues within individuals.

KEY RESULTS: In *L. argenteus* and *L. sulphureus*, pollen alkaloid concentrations were 11-35% of those found in other tissues. We detected no alkaloids in *L. bakeri* pollen, though they were present in other tissues. Alkaloid concentrations were not strongly correlated among tissues within individuals. We detected fewer alkaloids in pollen compared to other tissues and pollen contained no unique alkaloids.

CONCLUSIONS: Our results are consistent with the hypothesis that, in these pollen-rewarding species, pollen secondary chemistry may reflect the need to attract and reward pollinators more than the need to defend pollen from herbivory.

INTRODUCTION

Plant secondary compounds mediate interactions between plants and a diverse array of other organisms. Distasteful, repellant, and toxic secondary compounds are characteristic of plant defense against herbivory (Ehrlich and Raven, 1964). While individual secondary compounds often target certain species or guilds of interaction partners (Ehrlich and Raven, 1964; Agrawal and Weber, 2015), the expression of plant secondary compounds may also affect plant interactions with non-target species (Strauss and Armbruster, 1997; Strauss and Irwin, 2004; Theis et al., 2007; Kessler and Halitschke, 2009). For example, some floral volatiles that deter ants that steal nectar from flowers and damage the gynoecium without pollinating can also deter pollinators (Galen et al., 2011). Secondary compounds occur not only in leaves, stems, and roots, but also in seeds, fruit, flowers, and floral rewards, including nectar and pollen (McKey, 1974; Cipollini and Levey, 1997; Adler, 2000). Optimal Defense Theory (ODT, McKey, 1974; Rhoades, 1979) predicts that reproductive structures and tissues should be among the most well defended plant parts due to their high fitness values. Indeed, studies comparing secondary compound allocation between vegetative and reproductive tissues in rockcress (*Boechera stricta*; Keith and Mitchell-Olds, 2017), wild parsnip (*Pastinaca sativa*; Zangerl and Rutledge, 1996), and lungwort (*Lobaria scrobiculata*; Asplund et al., 2010) have found that secondary compounds are disproportionately allocated to reproductive tissues. Further, reproductive tissues may exhibit not only higher constitutive defenses, but also a greater ability to induce defense following damage compared to vegetative tissues (Zangerl and Rutledge, 1996; Keith and Mitchell-Olds, 2017), resulting in reduced herbivore damage relative to other tissues (Zangerl and Rutledge, 1996; Asplund et al., 2010).

Most studies that have measured the secondary chemistry of floral rewards have focused on nectar, finding that nectar secondary compounds tend to either mirror or represent a subset of the secondary compounds found in vegetative and floral tissues, but are typically present at considerably lower concentrations (Manson et al., 2012; Cook et al., 2013). Because nectar usually serves solely as a reward, it is not surprising that concentrations of defense related compounds are lower in nectar than in other plant tissues. Pollen serves two very different functions in many plants – as carriers of gametes and as a pollinator reward. In plants that offer both pollen and nectar as rewards, defending pollen from non-pollinating pollen consumers should be advantageous, and selection for well defended pollen follows from ODT (McKey, 1974; Rhoades, 1979). Consistent with this hypothesis, in some plants that offer nectar as a reward, the concentrations of secondary compounds in pollen far exceed those in nectar and may be comparable to those found in vegetative tissues (Palmer-Young et al., 2018). However, approximately 20,000 nectarless plant species offer pollen as the sole reward to pollinators (Willmer, 2011), and little is known about the pollen secondary chemistry in these pollen rewarding species, or about the variation in pollen chemistry among pollen-rewarding species (Parachnowitsch and Manson, 2015). Hereafter, we refer to plants that produce no nectar and only offer pollen as a reward to pollinators as pollen-rewarding plants. While potentially advantageous in defense against non-pollinating pollen consumers, chemically defended pollen may also come with the ecological cost of deterring pollinators in search of pollen rewards, presenting a conflict between the gamete and reward roles of pollen for pollen-rewarding species. How pollen-rewarding plants resolve the conflict between the pressure to defend pollen vs. enticing and rewarding pollinators has received little empirical attention within and across plant genera.

Lupines (*Lupinus spp.*, Fabaceae) are nectarless, pollen-rewarding, and primarily bee pollinated (though some may be capable of autogamous self-pollination) (Dunn, 1956; Gori, 1989), and thus represent ideal plants to assess patterns of secondary compound expression within pollen relative to a whole-plant context. Lupines produce quinolizidine and/or piperidine alkaloids, secondary compounds which are deterrent and toxic to some invertebrate herbivores and which may be toxic and/or teratogenic to ungulates (Dolinger et al., 1973; Panter et al., 1998; Lee et al., 2007a; Kozłowski et al., 2017). Only two studies, to our knowledge, have measured lupine pollen secondary chemistry, both finding that alkaloid concentrations in seeds, leaves, and/or flowers exceeded those in pollen (Detzel and Wink, 1993; Arnold et al., 2014). Detzel and Wink (1993) compared alkaloid concentrations from a composite sample within and between tissue types in *Lupinus polyphyllus*, and Arnold et al. (2014) only reported a single value of pollen alkaloid concentration from a composite sample of *Lupinus mutabilis* and did not report concentrations in other tissues. In Detzel and Wink (1993) pollen alkaloid concentrations were lower than other tissues tested, approx. 20 and 3.5% of the concentrations reported in flowers and leaves, respectively.

As alkaloids are broadly toxic to invertebrates, many lupine alkaloids may be toxic or deterrent to bees in sufficient concentrations, though to date only a few lupine alkaloids have been assessed for toxicity in bees (Detzel and Wink, 1993). Because lupines use pollen as their sole pollinator reward, and are entirely reliant on bee pollinators for reproduction, they may experience selection against the use of alkaloids to defend their pollen from non-pollinating pollen consumers such as beetles and lepidopteran larvae. Alternatively, alkaloid-rich pollen could be selected for if selection to defend pollen from non-pollinating pollen consumers overwhelms selection to attract and reward pollinators or is important to reduce over-collection

of pollen by bee pollinators (i.e., pollen that is lost in corbicular pollen loads and not available for pollination).

To determine whether the lower concentrations of secondary compounds in pollen relative to other plant tissues is common among species in the pollen-rewarding genus *Lupinus*, we compared the alkaloid profiles of pollen to other above-ground tissues in three Western North American *Lupinus* species: *Lupinus argenteus* Pursh, *Lupinus bakeri* Greene subsp. *amplus* Fleak and Dunn, and *Lupinus sulphureus* Douglas ex. Hook var. *sulphureus*. We hypothesized that the above-ground tissues, including pollen, would contain quinolizidine and/or piperidine alkaloids, based on prior studies in other *Lupinus* species (Detzel and Wink, 1993; Arnold et al., 2014), but that concentrations would be lower in the pollen relative to other tissues. We also hypothesized that the alkaloid profiles of pollen would be qualitatively similar to other above ground tissues. This study provides much needed empirical insight into the secondary chemistry of pollen and tests predictions about its evolutionary ecology in pollen-rewarding plants.

METHODS

Study System

The ranges of the three species included in our study span a broad swath of western North America from British Columbia to Washington State and Oregon (*L. sulphureus*), to Colorado and New Mexico (*L. bakeri*) and across all of the western United States and into northern Mexico (*L. argenteus*). This trio of species was ideal for our study because they cover a broad geographic range but share a common reward strategy (nectarless and pollen-rewarding) (Dunn,

1956), are pollinated by similar assemblages of insects (medium to large bodied bees including *Bombus* spp., *Andrena* spp., *Megachile* spp., and *Osmia* spp.) (Dunn, 1956; Heiling, pers. obs.), and share similar herbivores (primarily lycaenid butterflies and grazing mammals) (Dolinger et al., 1973; Severns, 2003).

Plant Material

We collected *L. argenteus* and *L. bakeri* samples from four and two sites, respectively, in and around the Rocky Mountain Biological Laboratory (RMBL), Gothic, Colorado, United States, and *L. sulphureus* from one site near Pendleton, Oregon, United States (Table 4.1). For all species, we collected single flowering stems from 6-7 plants at each site. Following collection, samples were separated into leaf, stem, flower, and pollen. For *L. argenteus* and *L. bakeri* we included only the corolla and gynoecium in flower samples, excluding the androecium. Due to minor differences in sampling protocols, *L. sulphureus* flower samples included some material from the androecium. For all three species, pollen samples consisted of granular pollen, no anther material was included. At each site, we collected and pressed a voucher specimen (Table 1). The *L. argenteus* and *L. bakeri* specimens are retained at the RMBL Herbarium, and the *L. sulphureus* specimen is retained at the Intermountain Herbarium (UTC) at Utah State University. Geographical information for sites and voucher numbers can be found in Table 4.1.

Chemicals and Reagents

We purchased ammonium hydroxide, sodium sulfate, and chloroform from Fisher Scientific (Pittsburgh, Pennsylvania, United States), Baker (Phillipsburg, New Jersey, United

States), and Mallinckrodt Baker (Paris, Kentucky, United States) respectively; caffeine and sparteine from Sigma-Aldrich Chemical Co. (St. Louis, Missouri and Milwaukee, Wisconsin, United States); lupanine from Biomedical Research Co. (Los Angeles, California, United States.); and D- α -Isolupanine perchlorate from Koch-Light Laboratories Ltd. (Colnbrook, Bucks, England).

Alkaloid Extraction

All plant material was air dried and ground to pass through a 2 mm screen. Once dry, we weighed a measured quantity into a 16 mL screw-top glass test tube for extraction. In brief, the plant material was extracted by mechanical rotation using the Rugged Rotator (Glas-Col LLC, Terre Haute, Indiana, United States) with a mixture of 1 N HCl (4.0 mL) and CHCl₃ (4.0 mL) for 15 min (as reported in Lee et al. 2007a). Following extraction, we centrifuged the samples (5 min) and removed the aqueous layer. To each test tube containing plant material and CHCl₃ we added an additional 2.0 mL of 1 N HCl, then repeated the extraction by mechanical rotation (15 min), centrifugation, and removal of the aqueous layer. The aqueous portions were combined in clean 16 mL screw-top glass test tubes. We adjusted their pH to 9.0-9.5 with concentrated NH₄OH and then extracted the basic solution twice with CHCl₃, first with 4.0 mL and then with 2.0 mL. These solutions were combined and filtered through anhydrous Na₂SO₄ into clean 16 mL screw-top glass test tubes and the solvent was evaporated under N₂ at 60°C. After reconstituting the extracted alkaloid fraction with a measured volume of methanol containing 1.3 μ g/mL caffeine (an internal standard), we transferred ~1 mL to 1.5 mL GC autosample vials for GC/FID or GC/MS analysis.

GC/FID Analysis

All samples were analyzed by GC/FID using a Shimadzu GC-2010 gas chromatograph (Shimadzu, Kyoto, Japan) equipped with a Shimadzu AOC-20i autosampler, a J&W DB-5 column (30 m x 0.32 mm, 0.25 μ m film thickness; J&W Scientific, Folsom, California, United States) and a flame ionization detector (FID). Samples were injected (1.0 μ L) splitless at 250°C using helium as the carrier gas at a constant flow rate of 2.0 mL/min. The column oven temperature program started at 100°C for 1 min; increased to 200°C at 50°C/min; increased to 260°C at 5°C/min; increased to 320°C at 50°C/min; and held at 320°C for 8.8 min for a total run time of 25 min. To calculate alkaloid concentrations, we used an eight-point sparteine standard curve (4-500 μ g/mL). Alkaloid concentrations are expressed as relative μ g of sparteine as standards were not available for all alkaloids.

GC/MS Analysis

We performed GC/MS analysis as previously reported in Lee et al. (2007a). In brief, we analyzed a minimum of one representative sample from each locality from the representative taxa by GC/MS using a Finnigan MAT GCQ (Finnigan MAT, San Jose, California, United States) equipped with a split/splitless injector and a DB-5MS (30 m x 0.25 mm; J&W Scientific, Folsom, California, United States) column. The injection port temperature was 250°C and was operated in the splitless mode. The split vent flow rate was 50 mL/min and purged after 0.80 min. The oven temperature was 100°C for 1 min; 100-200°C at 40°C/min; 200-275°C at 5°C/min; and then held at 275°C for 1.5 min. We used electron impact ionization (EI) at 70 eV with an ion source temperature of 200°C.

Alkaloid Identification

Alkaloid identification was performed as previously reported in Lee et al. (2007a). In brief, we identified six individual alkaloids from authenticated (MS, NMR) samples of ammodendrine, anagyrine, and thermopsine from the alkaloid collection of the Poisonous Plants Research Laboratory and from commercially obtained standards (sparteine, lupanine, and D- α -isolupanine). We determined the yet to be identified alkaloids from correlation of measured retention times (RT) to retention indices (RI) calculated by linear extrapolation from RI values generated from known standards and assigned RI numbers from the literature and their electron ionization (EI) mass spectra (Wink et al., 1995). In addition, alkaloid identification was further supported by correlation of measured relative retention times (RR_t) to lupanine RT and EI mass spectra reported in the literature (Kinghorn and Balandrin, 1984).

Statistical Analyses

Total alkaloid concentrations by tissue and site – We conducted all analyses in R version 3.5.1 (R Core Team, 2018). To compare total alkaloid concentrations across tissue types in each species, we used linear mixed effects models (LMM) with tissue type (leaf, flower, stem, and pollen) and site as fixed effects (for *L. sulphureus*, which came from a single site, tissue was the only fixed effect), an interaction between fixed effects of site and tissue, and individual as a random effect. The secondary chemistry profiles of lupine are known to vary systematically between sites (Carey and Wink, 1994; Muzquiz et al., 1994; Adler and Kittelson, 2004; Cook et al., 2018). While the primary goal of our study was to compare alkaloid patterns between tissues within individuals, we were still interested in capturing any between site variation in the populations that we studied. Hence, we included site as a fixed (rather than a random) effect. We

used the *lme4* package (Bates et al., 2014) to construct all LMMs, and fit these models by restricted maximum likelihood (REML) with Kenward-Roger approximations for degrees of freedom. We used the *lmerTest* package (Kuznetsova et al., 2015) to perform inference tests (ANOVA) on LMMs. For post-hoc significance tests on the main effect of tissue type, we performed Tukey Contrasts using the *emmeans* package (Lenth, 2018).

Between-tissue correlations of total alkaloid concentrations – To evaluate whether total alkaloid concentrations were correlated across tissue types within plants, we performed correlation analyses for each site-species combination. We used the function *rcorr()* in the *Hmisc* package (Harrell Jr and Harrell Jr, 2018) to construct correlation matrices with Spearman's rho rank correlation coefficients. Although it resulted in smaller sample sizes, we were unable to pool data across sites because of significant tissue $\times$ site interactions in our LMMs (see Results).

Alkaloid compositional analysis – To characterize the tissue-specific alkaloid profiles for each species, we performed non-metric multidimensional scaling (NMDS) on the alkaloid composition and concentration matrices (Bray-Curtis dissimilarity index) using the *metaMDS()* function in the *vegan* package (Cornille et al., 2012; Oksanen et al., 2013). *Lupinus argenteus* populations exhibit a variety of chemotypes (Wink and Carey, 1994). Pooled data from all four *L. argenteus* sites suggested two distinct sub-groups (Table 4.2). Accordingly, we divided the *L. argenteus* data into two separate NMDS analyses, one including data from the Gothic and Baldy sites representing one chemotype and another including data from the Elko and 401 sites representing another chemotype.

RESULTS

Alkaloid Identification

We identified 15 alkaloids and found one unknown alkaloid in our samples across all tissue types (Table 4.2 and Appendix A. S4.1-S4.3, see the Supplementary Data with this article), with 15 occurring in *L. argenteus*; four (including the unknown) occurring in *L. bakeri*; and three occurring in *L. sulphureus*. All taxa yielded quinolizidine alkaloids (typical of lupines), and *L. bakeri* yielded one piperidine alkaloid (ammodendrine). *Lupinus argenteus* and *L. sulphureus* pollen contained only a subset of the alkaloids identified in other above-ground tissues, and no alkaloids were unique to pollen (Table 4.2).

Total Alkaloid Concentrations by Tissue and Site

The tissue $\times$ site interaction term was significant in the initial models for *L. argenteus* and *L. bakeri* using pooled data from all of the sites for each species ($F_{9,66} = 5.46$, $P < 0.0001$ and $F_{3,36} = 3.29$, $P = 0.032$, respectively). Since we were interested in the patterns of alkaloid concentrations within individuals, not between sites, we ran separate models for each site.

Total alkaloid concentrations differed significantly by tissue type for all *L. argenteus* sites (Baldy: $F_{3,15} = 8.77$, $P = 0.0013$; Elko: $F_{3,18} = 11.74$, $P = 0.0002$; 401: $F_{3,18} = 35.44$, $P < 0.0001$; Gothic: $F_{3,15} = 59.31$, $P < 0.0001$), for all *L. bakeri* sites (Upper Loop: $F_{3,18} = 31.12$, $P < 0.0001$; Kapuchion: $F_{3,18} = 27.52$, $P < 0.0001$), and for *L. sulphureus* ($F_{3,18} = 17.21$, $P < 0.0001$). Mean pollen alkaloid concentrations were 17-35% of those in other tissues in *L. argenteus* (Fig. 4.1A-D), and 11-28% of those in other tissues in *L. sulphureus* (Fig. 4.1G). We did not detect any alkaloids in *L. bakeri* pollen (Fig. 4.1E&F; Table 4.2). Tukey contrasts for tissue-by-tissue concentration comparisons are reported in Table 4.3.

Between-Tissue Correlations of Total Alkaloid Concentrations

Correlations in total alkaloid concentrations among tissue types ranged from -0.93 to 0.93 across all sites and species, but few of the correlations were statistically significant (Fig. 4.2). For *L. argenteus*, flower alkaloid concentrations were significantly negatively correlated with stem alkaloids at 401 ($\rho = -0.93$, $P = 0.0025$, Fig. 4.2B lower) while pollen and flower alkaloid concentrations were positively correlated at Baldy but negatively correlated at Elko ($\rho = 0.89$, $P = 0.0068$; $\rho = -0.82$, $P = 0.0234$; Fig. 4.2A upper and lower, respectively). In *L. bakeri*, the only significant correlation was between leaf and stem alkaloid concentrations at Kapuchion ($\rho = 0.86$, $P < 0.05$, Fig. 4.2C upper). Likewise, in *L. sulphureus* the only significant correlation was between pollen and stem alkaloid concentrations ($\rho = 0.93$, $P < 0.01$; Fig 4.2D). There were eight other tissue-tissue correlations with a $|\rho| > 0.5$, but none of these were statistically significant.

Alkaloid Compositional Analysis

The ordination analyses suggest differentiation between pollen alkaloid profiles and those of the other tissues (Fig. 4.3). Pollen alkaloid profiles were distinct from both flower and leaf profiles for all three species (Figs. 4.3, 4.4 and 4.5; note that pollen is not included in the *L. bakeri* plot in Fig. 4.3 because there were no detectable alkaloids in any pollen samples in this species). Flower, leaf, and stem tissues also formed distinct clusters from one another in *L. argenteus* and *L. sulphureus* but were not clearly differentiable in *L. bakeri* (Fig. 4.3). The later may be driven by an extreme flower profile observation (Fig. 4.3 upper right of panel C), but a Grubbs Test (using the R package ‘*outliers*’; Komsta, 2011) did not identify this value as an outlier ($G = 2.684$; $P = 0.158$).

DISCUSSION

Pollen is critical to reproduction in most flowering plants, and pollen secondary chemistry should shape and be shaped by interactions with pollen foragers. However, surprisingly few studies have measured pollen secondary chemistry in a whole-plant context (reviewed in Irwin et al. 2014) and even fewer in pollen-rewarding plant species (but see Detzel and Wink 1993, Arnold et al. 2014). We predicted that alkaloid concentrations would be lower in pollen than other tissue types but that the composition (identity and concentration) of alkaloids in pollen would be qualitatively similar to those of other tissues in the three pollen-rewarding *Lupinus* species that we studied. Our results generally supported these predictions. We found that alkaloid concentrations were significantly lower in pollen relative to other tissues (Fig. 4.1), and that alkaloid concentrations were not strongly correlated among tissues within individuals (Fig. 4.2). The distinct clustering of pollen observed in Fig. 4.3 is likely primarily explained by differences in concentration relative to other tissues (Fig. 4.1, 4.4, and 4.5). While pollen contained only a subset of alkaloids observed in the other above-ground tissues, no alkaloids were unique to pollen (Table 4.2), and no alkaloids were present in higher concentrations in pollen than in other tissues (Appendix A. S4.1 and S4.3). Instead, alkaloids that tended to be lowest in concentration in other above-ground tissues were absent in pollen.

Floral parts are closely tied to plant reproductive success, and thus fitness (Strauss, 1997; Adler et al., 2001; Irwin et al., 2004; Kessler and Halitschke, 2009). ODT predicts that, of the tissues we tested, flowers and pollen should be the most well-defended as they are most closely tied to reproductive success (McKey, 1974; Rhoades, 1979; Kessler and Halitschke, 2009). Consistent with the predictions of ODT, total alkaloid concentrations in flower tissue were consistently as high as or higher than those of leaves or stems. The universally high

alkaloid concentration in leaves relative to other tissues is also consistent with the predictions of ODT, as the growing season for these species is brief (only 2-3 months at higher elevations), which may select for high investment in defense of leaf tissue. However, our results for pollen were not consistent with ODT but rather with the hypothesis that, due to its dual roles as gametophyte and pollinator reward, pollen should have lower concentrations of secondary compounds relative to other tissues in pollen rewarding plants.

While non-pollinating pollen consumers should select for defended pollen, Muth et al. (2016) found that *Bombus impatiens* allowed to forage on artificial flowers containing *Prunus avium* pollen treated with either sucrose, cellulose (control), or quinine (a defensive alkaloid) made fewer visits to and spent less time collecting pollen from quinine treated artificial flowers, and were more likely to switch to a novel artificial flower type than bees in the other two groups, indicating that the preferences of pollen-foraging pollinators may select against chemically defended pollen. While these experimental results are compelling, we know little about the secondary chemistry of pollen in pollen-rewarding plants.

In particular, many of the studies that have assessed pollen secondary chemistry have been limited to single species and single or few tissues, limiting the comparative value of these data (Kessler and Halitschke, 2009). Further, the majority of this work has focused on species that also provide other rewards, most commonly nectar (e.g., London-Shafir et al., 2003; Cook et al., 2013; Egan et al., 2018). Such species are not strictly pollen rewarding and so may be expected to chemically defend their pollen. Arnold et al. (2014) reported negative effects of the lupine alkaloid *D*-lupanine on production of male bumble bees (*Bombus terrestris*) in microcolonies fed exclusively on pollen containing 0.02 – 0.2% *D*-lupanine (syn. lupanine), while Detzel and Wink (1993) found that concentrations of the lupine alkaloid sparteine were

deterrent (ED₅₀) and toxic (LD₅₀) to honey bees when fed in sugar solutions at concentrations of 0.03 and 0.05%, respectively. Beyond these two alkaloids, we do not know whether or to what degree bees are sensitive to other lupine alkaloids, and this should be the subject of further study. The different animal taxa that plants interact with often vary in their sensitivities to a given secondary compound (e.g., Saunders et al., 1992; Galen et al., 2011). Pollinators, such as *Bombus*, may be more sensitive to defensive secondary compounds than the herbivores that have driven the evolution of the defenses (Roslin and Salminen, 2008; Kelly and Bowers, 2016; but see: Bernays et al., 2004). For example, while bees are sensitive to lupanine and sparteine at relatively low concentrations (Arnold et al., 2014; Detzel and Wink, 1993), Johnson and Bentley (1988) found that much higher concentrations of these same alkaloids were required to reduce growth and survivorship of herbivorous army worm (*Spodoptera eridania*) larvae. We suspect that many of the other alkaloids that we describe here may also have toxic or deterrent effects on bees in high enough concentrations.

The total alkaloid concentrations in pollen were variable among individual plants in our study (ranging from 0-1.1% dry mass), but they were very low overall (mean in *L. argenteus* and *L. sulphureus*: 0.27% ± 0.05 SE dry mass) relative to other tissues (Fig. 4.1, Appendix A. S4.1-4.3). We observed mean pollen alkaloid concentrations that fell within the ranges shown by Arnold et al. (2014) and Detzel and Wink (1993) to negatively affect bees fed exclusively on food (pollen and sugar, respectively) with these alkaloid concentrations. However, it seems unlikely that natural lupine pollen alkaloids concentrations would deter or intoxicate pollen foraging polylectic bees such as the *Bombus* spp. which are the primary pollinators of North American lupines (Dunn, 1956) because the concentrations that such bees are effectively exposed to will be lower than the population mean due to diet mixing. As bees collect pollen

from several individual plants, and polylectic bees collect pollen from several species, the high variability in alkaloid concentration and composition among individual plants may limit the exposure that a colony will experience to any one alkaloid. This should be contrasted with the more focused exposure to a given set of lupine alkaloids that an herbivore, which spends its entire juvenile stage on an individual plant (e.g., lepidopteran larvae), would experience. Further, as within-population variation in pollen alkaloid concentrations was fairly high (1-2 orders of magnitude), and bees have been demonstrated to use taste to selectively forage on pollen with lower alkaloid concentrations when given the choice (Muth et al., 2016), bees may limit their exposure to alkaloids by avoiding individuals that express higher alkaloids concentrations in their pollen. However, the oligolectic species which pollinate *Lupinus* species may experience much higher effective doses of lupine pollen alkaloids, suggesting an exciting avenue for future work. Interestingly, in sites near Gothic, CO, the Fabaceae specialist *Osmia iridis* does not collect lupine pollen, though the flowers are locally abundant in areas where they forage (Spear et al., 2016; Forrest and Chisholm, 2017). It is possible that, while low relative to other tissues, the alkaloids present in the pollen of *L. argenteus* are at high enough concentrations to deter these oligolectic and relatively small-bodied bees.

The overall lack of significant correlations between tissue alkaloid concentrations across all three species is, on the surface, surprising. While our knowledge of within-individual variation in secondary chemistry lags behind our understanding of variation among individuals, some studies have found correlations in secondary compound profiles across tissues within individuals (Adler et al., 2006, 2012). However, for all species and populations of *Lupinus* in our study, we found that, at most, only two tissue types were ever correlated. Further, there were no consistent trends in the tissue alkaloid correlations within or across species. Two factors may

limit the strength of these results. First, sample sizes were small ($N = 6-7$ individuals per site). This may mean that the type II error was high, and that we were unable to detect some true correlations, if they existed. Second, while we collected samples at roughly the same phenological stage at each site, we did not fully control for phenology. In general, relative chemical concentrations across tissues vary temporally as secondary compounds produced in one tissue are transported to other tissues (Lee et al., 2006), and as secondary compounds degrade with time (Wink and Witte, 1984). With these limitations in mind, a cautious interpretation of our results is that selection may act on the alkaloid concentrations of several or all of these tissues independently, consistent with the hypothesis that pollen foraging by pollinators could select for lower concentrations of defensive secondary compounds in pollen. Similar results were described by Kessler et. al. (2009) in the pollen-rewarding wild tomato (*Solanum peruvianum*), and echo patterns described in some studies of nectar secondary chemistry (Manson et al., 2012). These results suggest that the uncoupling of defensive secondary chemistry between rewards and other tissues may be common.

While alkaloid profiles of *Lupinus* species, as described here and by previous authors (e.g. Dolinger et al., 1973; Wink and Carey, 1994; Wink et al., 1995; Cook et al., 2009), are characteristically variable both within and among species and populations, two features were common to all populations and species in our study. First, pollen alkaloid concentrations were consistently and significantly lower than in other tissues, and second, pollen alkaloid composition represented only a subset of other above-ground tissues (Tables 4.2, Appendix A. S4.1, and S4.3). The simplified composition of alkaloids in pollen relative to other tissues may reflect either the lack of expression of these alkaloids in pollen or proportionally lower expression in pollen such that the concentrations fall below the detection threshold of our

analytical methods. Interestingly, of the two lupine alkaloids found in our samples and known to be toxic and deterrent to bees (Detzel and Wink, 1993; Arnold et al., 2014), lupanine was only present in pollen from two populations (Appendix A. S1 and S3) and we did not detect sparteine, or its stereoisomers (α -isosparteine and β -isosparteine), in the pollen of any of the species in our study (Tables 4.2 and Appendix A. S4.1-S4.3). Whether *Bombus* are as sensitive to the alkaloids that are more abundant in the *Lupinus* we sampled remains unclear. The absence of these compounds in the pollen that we sampled may reflect selection against their inclusion in pollen, possibly because of deterrent or toxic effects on pollinators. However, the apparent absence of individual alkaloids in our samples could be the result of small sample sizes, spatial or temporal variation in alkaloid expression (either due to phenotypic plasticity or population-genetic variation), or a combination of these factors.

CONCLUSIONS

Pollen-rewarding plant species may be engaged in a defense—attraction tug-of-war. The relative importance of selection on pollen chemistry via pollinator attraction vs. plant defense will depend on the magnitude of pollen limitation of plant reproduction as well as levels of pollen herbivory and their effect on plant fitness. We cannot yet rule out the possibility that pollen alkaloids, or lack thereof, may reflect pleiotropic effects of the production and transport of alkaloids across other tissues (Adler, 2000), selection via pollen's role as a gamete, or to reduce over-collection by pollen-harvesting bees (Parachnowitsch and Manson, 2015). However, the low concentration of alkaloids that we observed in the pollen of these three *Lupinus* species is consistent with the hypothesis that these species may experience selection against the use of alkaloids to defend their pollen from non-pollinating pollen consumers. Studies assessing

whether pollen secondary chemistry serves an adaptive function in these and other species are now needed. In light of these results, future studies should ask: (1) What is the relative importance of pollinator attraction vs. plant defense with respect to pollen loss and how might this vary across species and communities, (2) how might this differ for male and female components of plant fitness, and (3) is there evidence that selection for chemically defended pollen undermines the pollen-rewarding strategy? Answering these questions is an important next-step in understanding the patterns and implications of pollen secondary chemistry and the pollen-rewarding character state which is so widespread among the angiosperms.

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AUTHOR CONTRIBUTIONS

J.H., D.C., and R.I. designed the study. J.H. and D.C. carried out fieldwork. D.C. and S.L. carried out lab work. J.H. analyzed the data with the input of D.C. and R.I. J.H., D.C., S.L. and R.I. wrote the manuscript.

DATA ACCESSIBILITY

Data are archived with and available through Dryad, DOI: <https://doi.org/10.5061/dryad.586f011>

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Table 4.1. GPS coordinates, elevation (meters above sea level), and voucher identification numbers for *Lupinus* sites sampled. *Lupinus argenteus* and *L. bakeri* vouchers are housed in the herbarium of the Rocky Mountain Biological Laboratory (Global Unique Identifier: dcc3c0d4-53b3-4be4-9de7-a6c1d63736c5); *L. sulphureus* vouchers are housed in Intermountain Herbarium, Utah State University.

Species	Site	Latitude	Longitude	Elevation (masl)	Voucher ID
<i>Lupinus argenteus</i>	401	39.008381	– 107.02717	3306	RMBL0011502
	Gothic (GTH)	38.957441	– 106.9891	2872	NA
	Baldy (BDY)	38.977309	– 107.04126	3521	RMBL0011548
	Elko (ELK)	39.008124	– 107.05431	3278	RMBL0011503
<i>Lupinus bakeri</i>	Upper Loop (UPL)	38.88303	– 107.95976	2814	RMBL0011499
	Kapuchion (KAP)	38.90362	– 107.01328	2745	NA
<i>Lupinus sulphureus</i>	Pendelton (PEN)	45.593617	– 118.53918	1127	UTC247072

Table 4.2. Alkaloids identified in *Lupinus argenteus*, *L. bakeri*, and *L. sulphureus* tissues in samples from populations near Gothic, Colorado (*L. argenteus* and *L. bakeri*) and Pendleton, Oregon (*L. sulphureus*). Columns are arranged by species, variety, and site, respectively. Letters denote presence of a given alkaloid in samples of corresponding tissue (pollen: P, flower: F, leaf: L, stem: S). -- indicates the alkaloid was not detected; † indicates the alkaloid was detected in < 75% of samples.

	<i>Lupinus argenteus</i>				<i>L. bakeri</i>		<i>L. sulphureus</i>
	401	GTH	BDY	ELK	KAP	UPL	PEN
Ammodendrine	--	--	--	--	FLS	FLS	--
Anagryne	--	PFLS	PFLS	--	--	--	PFLS
Aphyllidine	--	FLS	--	--	--	--	--
Argyrolobine	--	P [†] FLS	--	--	--	--	--
5,6 Dehydrolupanine	--	FLS	PFLS	--	--	F [†] L [†]	FLS
11,12 Dehydrolupanine	FS [†]	--	--	PF [†] LS	--	--	--
5,6 Dehydro- α -isolupanine	FLS	--	--	P [†] FLS	--	--	--
Dihydroxyaphyllidine	--	FLS	--	--	--	--	--
3-Hydroxy-lupanine	--	--	P [†] F [†] L [†] S [†]	--	--	--	--
α -isolupanine	PFLS	--	--	PFLS	--	--	--
α -isosparteine	FLS [†]	--	--	F [†] LS [†]	--	--	--
β -Isosparteine	--	L	F [†] LS [†]	--	--	--	--
Lupanine	--	FLS	PFLS	--	--	F [†] L [†]	PFLS
Sparteine	--	FLS [†]	FLS [†]	--	--	--	--
Thermopsine	PFLS	--	--	PFLS	--	--	--
Unknown	--	--	--	--	F [†] L [†] S [†]	F [†] L [†] S [†]	--

Table 4.3. Tukey contrasts (p-values) from tissue-by-tissue alkaloid concentration comparisons. NS = Not significant, NA = No contrast performed due to lack of detectable alkaloids in pollen. Letters denote tissue - pollen: P, flower: F, leaf: L, stem: S.

Species	Site	P-F	P-L	P-S	F-S	F-L	L-S
<i>L. argenteus</i>	BDY	0.0011	0.0067	0.0445	NS	NS	NS
	ELK	0.0002	0.0152	NS	0.003	NS	NS
	401	<0.0001	<0.0001	NS	<0.0001	NS	0.001
	GTH	<0.0001	<0.0001	0.0002	0.0002	NS	0.005
<i>L. bakeri</i>	UPL	NA	NA	NA	NS	NS	0.0066
	KAP	NA	NA	NA	NS	0.0302	0.0174
<i>L. sulphureus</i>	PEN	<0.0001	0.0005	NS	0.0018	NS	0.0476

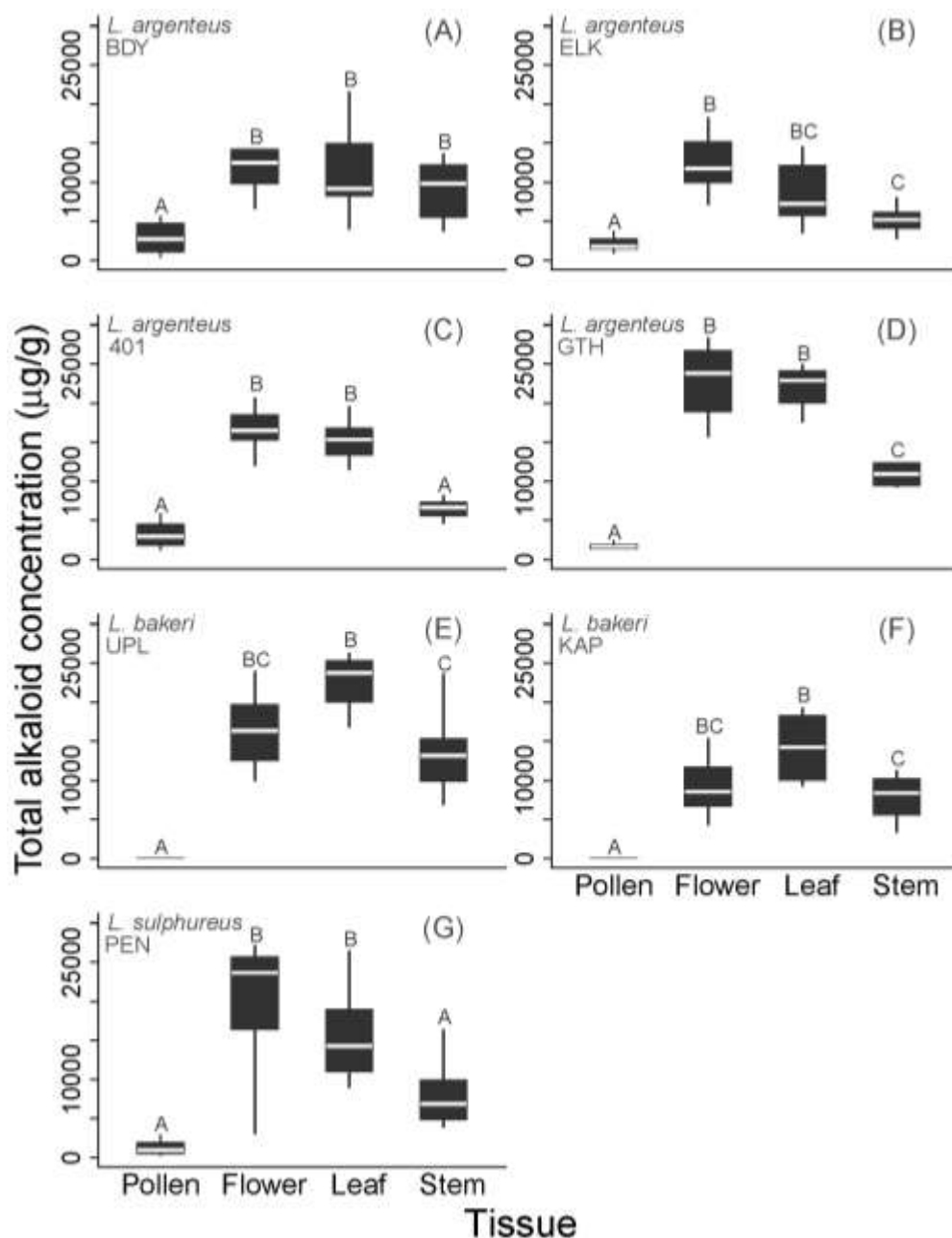


Figure 4.1. Total alkaloid concentrations (µg/g) in tissues of *Lupinus argenteus* from (A) Baldy, (B) Elko, (C) 401, and (D) Gothic, *Lupinus bakeri* from (E) Upper Loop and (F) Kapuchion, and (G) *Lupinus sulphureus* from Pendleton. Bars are boxplots, with lower and upper ends of boxes depicting the lower and upper quartiles, respectively. Solid bands indicate medians. Whiskers extend across the data range. Different uppercase letters above boxes indicate significant differences in alkaloid concentrations at $P \leq 0.05$ based on Tukey contrasts.

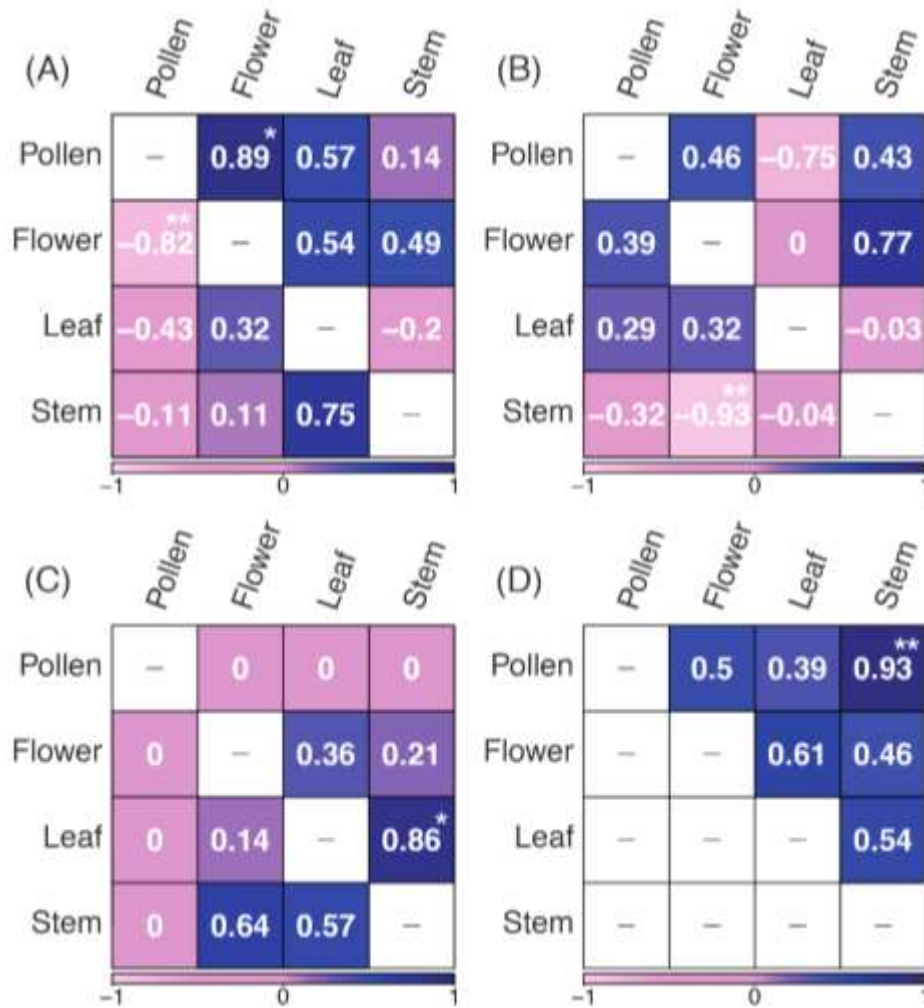


Figure 4.2. Correlograms of total alkaloid concentrations showing correlations among tissue types (pollen, leaf, flower, and stem) for (A & B) *Lupinus argenteus*, (C) *L. bakeri*, and (D) *L. sulphureus*. In panels A-C, two sites are shown per panel, A: Baldy (upper) and Elko (lower), B: Gothic (upper), 401 (lower); C: Kapuchion (upper), Upper Loop (lower). Correlation coefficients are Spearman's ρ rank. Asterisks indicate P-value ranges: * $P \leq 0.05$, ** $P \leq 0.01$.

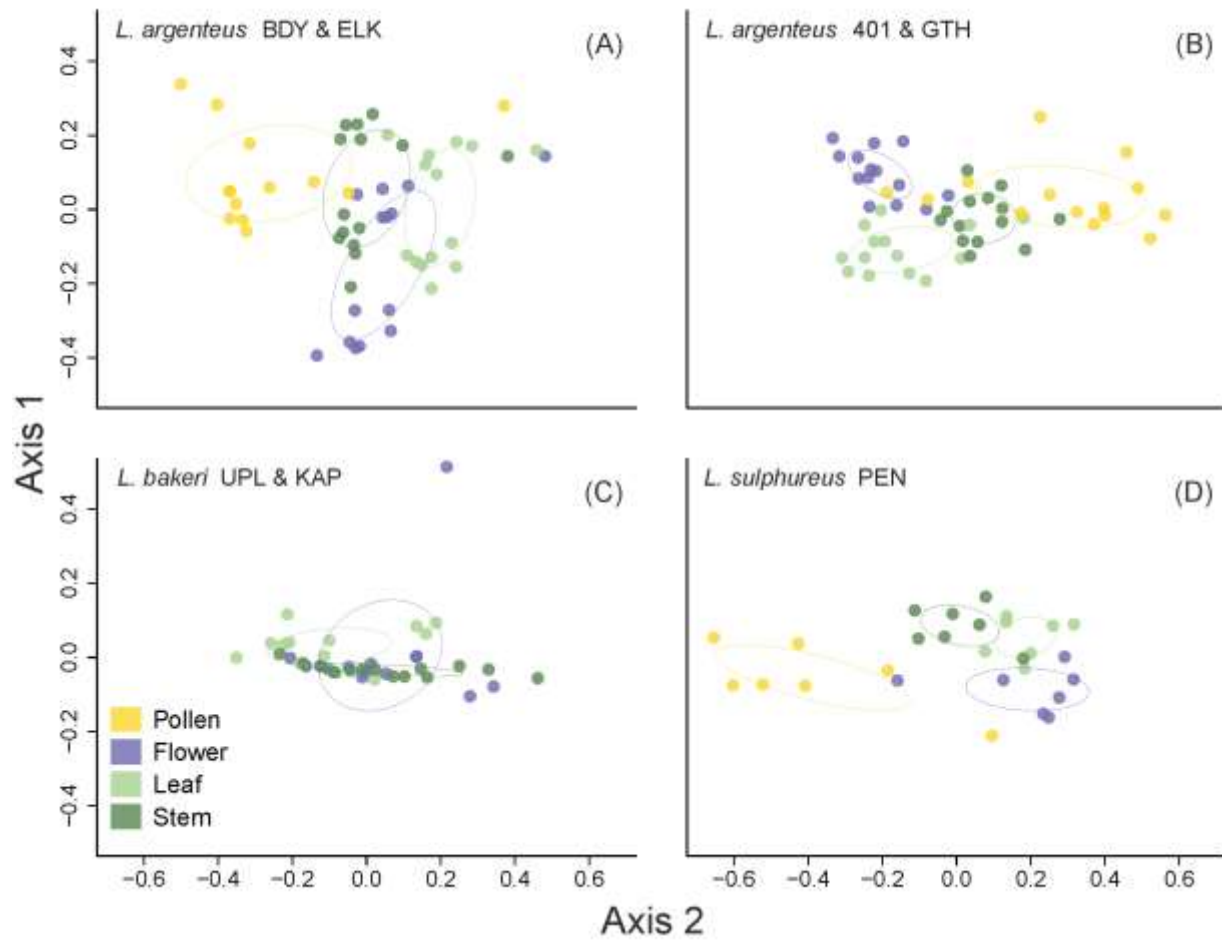


Figure 4.3. Non-metric multidimensional scaling (NMDS) ordination plots of alkaloid profiles across four tissue types (pollen, leaf, flower, and stem) of (A, B) *Lupinus argenteus* (stress = 0.158 and 0.092), (C) *L. bakeri* (stress = 0.041), and (D) *L. sulphureus* (stress = 0.049). Data for *Lupinus argenteus* are divided into two plots: (A) Gothic and Baldy sites and (B) Elko and 401 sites. Pollen is not included in the plot for *L. bakeri* because we did not detect any alkaloids in the pollen samples from this species. Ellipses are SD.

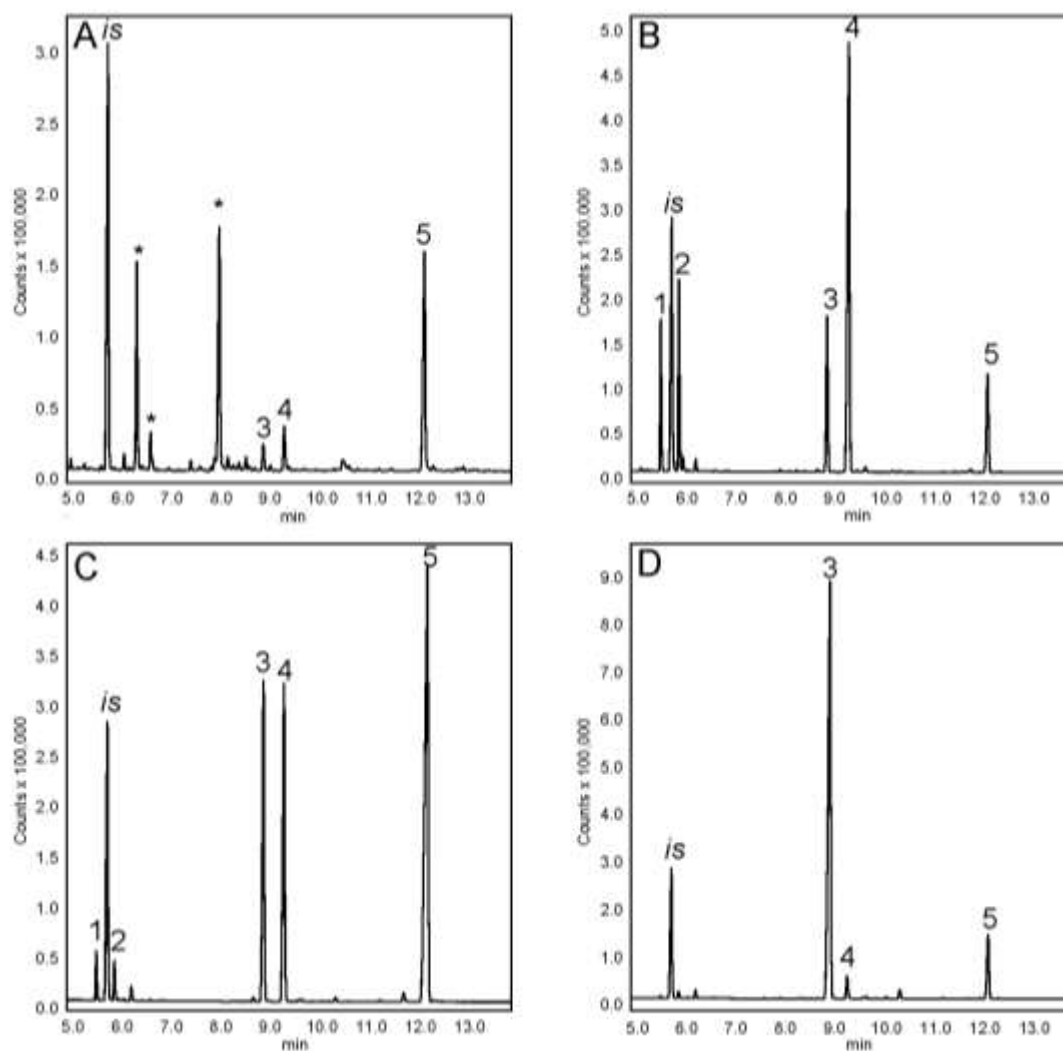


Figure 4.4. GC-FID chromatogram representative of *Lupinus argenteus* collected near Gothic, Colorado (BDY): (A) pollen, (B) leaves, (C) flowers, and (D) stems. Peak 1: sparteine; 2: β-isosparteine; 3: 5,6 dehydrolupanine; 4: lupanine; 5: anagyrene; is: internal standard (caffeine); *: unidentified pollen-specific analytes, found in all pollen samples.

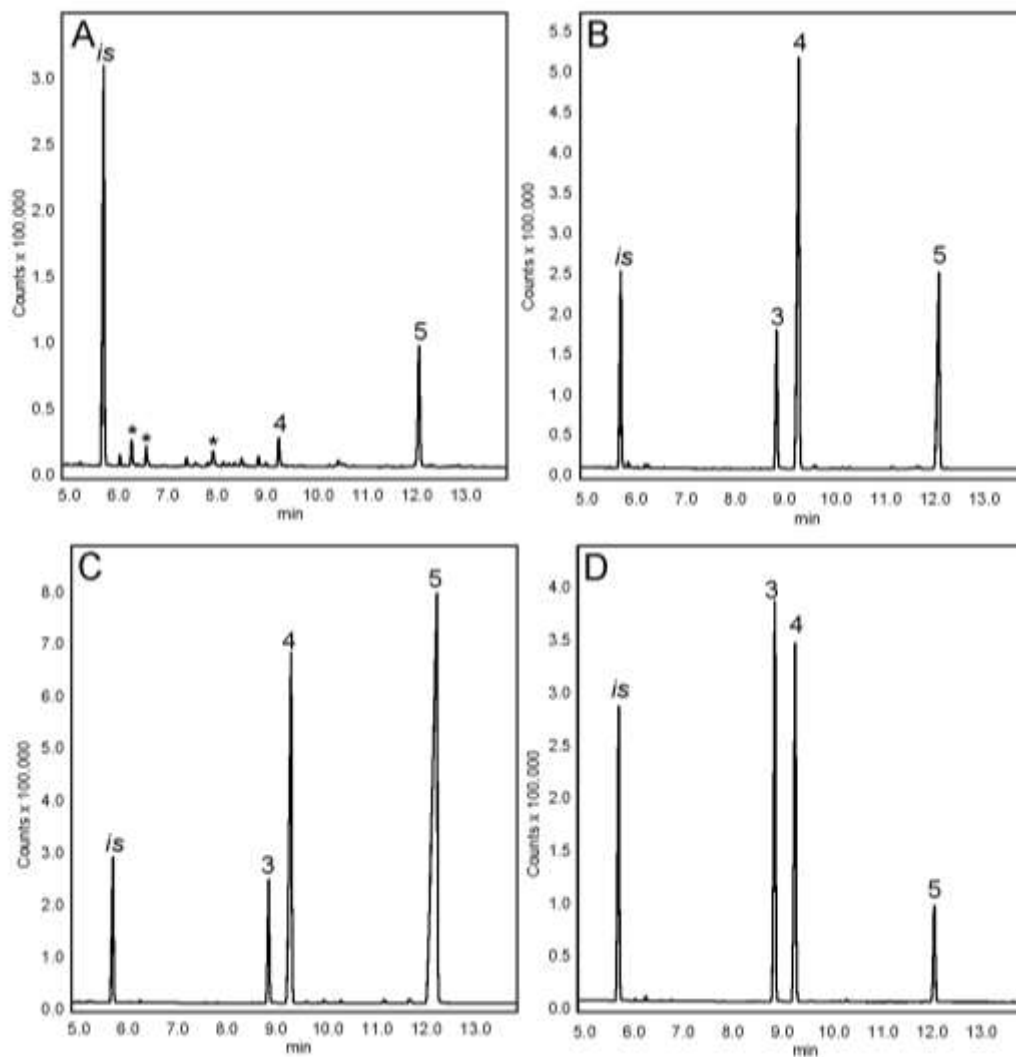


Figure 4.5. GC-FID chromatogram representative of *Lupinus sulphureus* collected near Pendleton, Oregon. (A) pollen, (B) leaves, (C) flowers, and (D) stems. Peak 3: 5,6 dehydrolupanine; 4: lupanine; 5: anagryrine; *is*: internal standard (caffeine); *: unidentified pollen-specific analytes, found in all pollen samples.

APPENDICES

Appendix A

Table S1.1 Latitude, longitude, and elevation for all sites used from 2014-2016.

Site	Latitude	Longitude	Elevation (masl)
Kebler Pass	38.86558	-107.10528	3086.40
Poverty Gulch	38.95615	-107.07713	2917.55
Washington Gulch	38.95997	-107.03379	3149.80

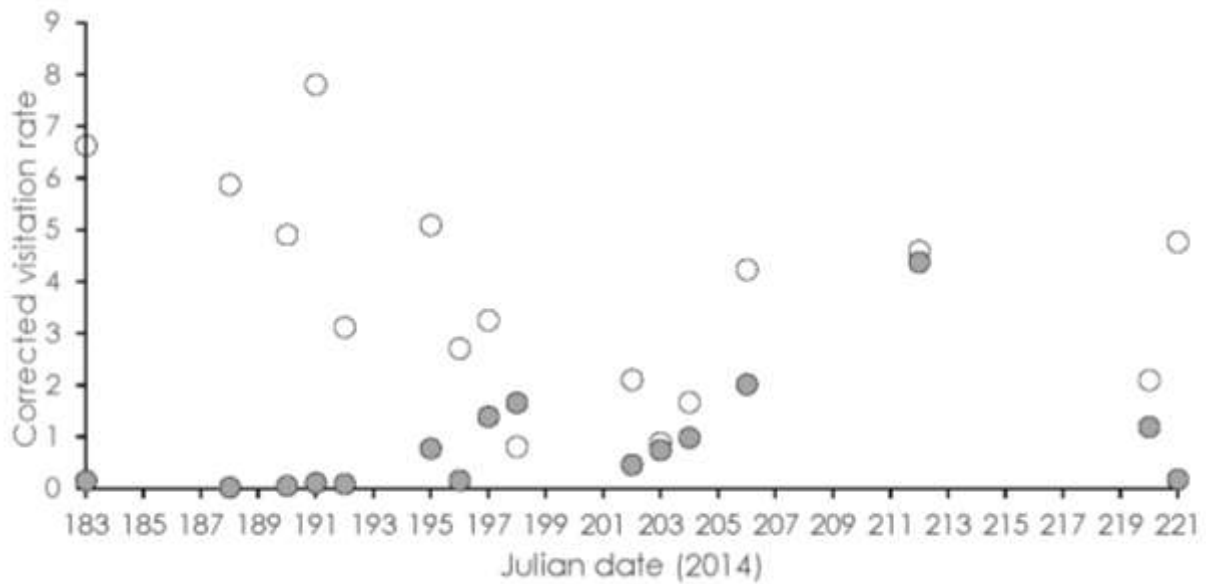


Figure S1.1 Corrected visitation rates (visits observed per minute effort) for pollinators (open circles) and robbers (filled circles) for each sampling date.

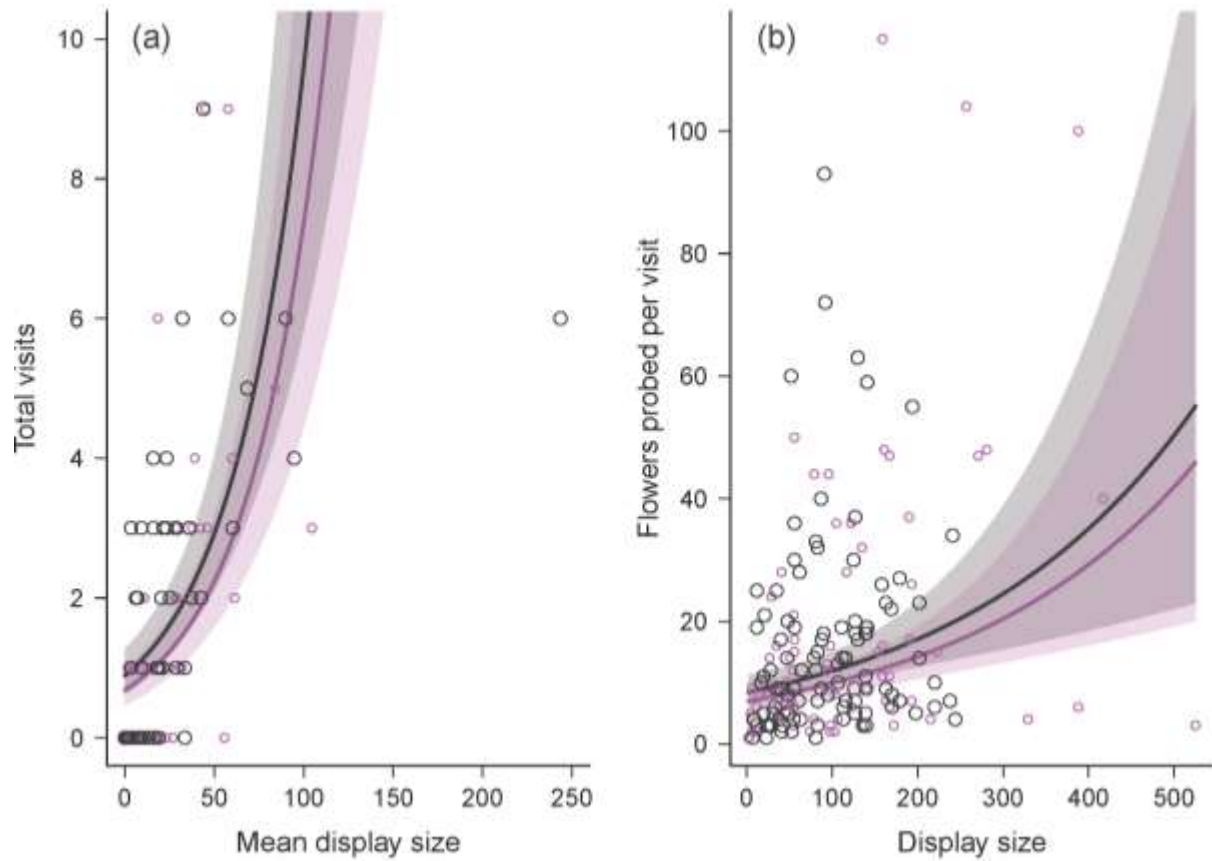


Figure S2.1 (a) Total number of pollinator visits observed per plant throughout the season. (b) Mean visitation sequence length. Black lines are the fitted curves for the nectar-supplemented plants, violet line is the fitted curve for control plants. Large black circles are datapoints for nectar-supplemented plants, small violet circles are datapoints for control plants. Bands represent 95% CI.

Table S4.1 Concentrations ($\mu\text{g/g}$) of alkaloid species observed in *Lupinus argenteus* tissues. Subscripts denote the number of individuals that a given alkaloid was detected in out the total number of individuals sampled. Error estimates are $\pm 1\text{SE}$. N/D = Not detected

	Pollen			
	401	Gothic	Baldy	Elko
Ammodendrine	N/D	N/D	N/D	N/D
Anagryne	N/D	1374.97 _{7/7} (± 230.66)	1555.61 _{5/6} (± 470.77)	N/D
Aphyllidine	N/D	N/D	N/D	N/D
Argyrolobine	N/D	478.75 _{2/7} (± 99.42)	N/D	N/D
5,6 Dehydrolupanine	N/D	N/D	390.23 _{5/6} (± 190.57)	N/D
11,12 Dehydrolupanine	N/D	N/D	N/D	N/D
5,6 Dehydro- α - isolupanine	N/D	N/D	N/D	565.32 _{1/7}
Dihydroxyaphyllidine	N/D	N/D	N/D	N/D
3-Hydroxy-lupanine	N/D	N/D	2414.25 _{1/6}	N/D
α -Isolupanine	2489.49 _{7/7} (± 1099.41)	N/D	N/D	1861.84 _{7/7} (± 579.18)
α -Isosparteine	N/D	N/D	N/D	N/D
β -Isosparteine	N/D	N/D	N/D	N/D
Lupanine	N/D	N/D	875.59 _{6/6} (± 326.33)	N/D
Sparteine	N/D	N/D	N/D	N/D
Thermopsine	1540.67 _{7/7} (± 328.26)	N/D	N/D	845 _{7/7} (± 326.31)
Unknown	N/D	N/D	N/D	N/D
	Flower			
	401	Gothic	Baldy	Elko
Ammodendrine	N/D	N/D	N/D	N/D
Anagryne	N/D	11999.34 _{7/7} (± 2691.78)	3823.16 _{7/7} (± 893.53)	N/D
Aphyllidine	N/D	1255.08 _{7/7} (± 363.36)	N/D	N/D
Argyrolobine	N/D	4657.65 _{7/7} (± 1328.59)	N/D	N/D
5,6 Dehydrolupanine	N/D	632.92 _{6/7} (± 136.89)	2662.94 _{7/7} (± 581.06)	N/D

Table S4.1 (continued)

	428.76 _{6/7} (±194.04)	N/D	N/D	747.88 _{7/7} (±91.80)
11,12 Dehydrolupanine				
5,6 Dehydro- α - isolupanine	976.018 _{7/7} (±282.75)	N/D	N/D	802.95 _{7/7} (±176.72)
Dihydroxyaphyllidine	N/D	3337.24 _{7/7} (±883.47)	N/D	N/D
3-Hydroxy-lupanine	N/D	N/D	10259.13 _{1/7}	N/D
α -Isolupanine	6266.82 _{7/7} (±773.95)	N/D	N/D	6725.78 _{7/7} (±662.29)
α -Isosparteine	1329.64 _{7/7} (±71.58)	N/D	N/D	409.27 _{4/7} (±161.17)
β -Isosparteine	N/D	N/D	220.10 _{4/7} (±34.80)	N/D
Lupanine	N/D	254.18 _{7/7} (±39.39)	4031.31 _{7/7} (±1350.68)	N/D
Sparteine	N/D	329.90 _{6/7} (±102.42)	225.92 _{6/7} (±41.42)	N/D
Thermopsine	8330.89 _{7/7} (±1051.65)	N/D	N/D	3974.85 _{7/7} (±718.04)
Unknown	N/D	N/D	N/D	N/D

	Leaf			
	401	Gothic	Baldy	Elko
Ammodendrine	N/D	N/D	N/D	N/D
Anagryne	N/D	2235.57 _{7/7} (±697.97)	735.92 _{6/7} (±169.58)	N/D
Aphyllidine	N/D	3489.81 _{7/7} (±502.94)	N/D	N/D
Argyrolobine	N/D	5134.94 _{7/7} (±630.77)	N/D	N/D
5,6 Dehydrolupanine	N/D	2181.52 _{6/7} (±491.34)	1285.26 _{7/7} (±370.18)	N/D
11,12 Dehydrolupanine	N/D	N/D	N/D	141.54 _{4/7} (±15.13)
5,6 Dehydro- α - isolupanine	501.21 _{7/7} (±119.88)	N/D	N/D	353.60 _{6/7} (±94.86)
Dihydroxyaphyllidine	N/D	2043.41 _{7/7} (±531.04)	N/D	N/D
3-Hydroxy-lupanine	N/D	N/D	4134.31 _{1/7}	N/D

Table S4.1 (continued)

α -Isolupanine	6141.76 _{7/7} (± 660.13)	N/D	N/D	4940.28 _{7/7} (± 788.52)
α -Isosparteine	6147.98 _{7/7} (± 1021.95)	N/D	N/D	2588.17 _{6/7} (± 730.16)
β -Isosparteine	N/D	585.78 _{7/7} (± 47.38)	1208.48 _{7/7} (± 255.43)	N/D
Lupanine	N/D	3117.09 _{7/7} (± 393.45)	5496.22 _{7/7} (± 1135.44)	N/D
Sparteine	N/D	2298.60 _{7/7} (± 596.41)	1647.95 _{6/7} (± 900.72)	N/D
Thermopsine	2461.83 _{7/7} (± 271.49)	N/D	N/D	1192.82 _{7/7} (± 437.02)
Unknown	N/D	N/D	N/D	N/D
Stem				
	401	Gothic	Baldy	Elko
Ammodendrine	N/D	N/D	N/D	N/D
Anagryne	N/D	4319.97 _{7/7} (± 1595.49)	642.99 _{7/7} (± 130.89)	N/D
Aphyllidine	N/D	860.66 _{6/7} (± 139.06)	N/D	N/D
Argyrolobine	N/D	1117.76 _{7/7} (± 186.37)	N/D	N/D
5,6 Dehydrolupanine	N/D	4565.97 _{7/7} (± 300.54)	5710.57 _{7/7} (± 1403.86)	N/D
11,12 Dehydrolupanine	520.26 _{5/7} (± 86.41)	N/D	N/D	139.44 _{2/7} (± 6.8)
5,6 Dehydro- α - isolupanine	1511.56 _{7/7} (± 334.17)	N/D	N/D	909.16 _{7/7} (± 204.89)
Dihydroxyaphyllidine	N/D	681.76 _{6/7} (± 133.15)	N/D	N/D
3-Hydroxy-lupanine	N/D	N/D	6009.62 _{1/7}	N/D
α -Isolupanine	3325.75 _{7/7} (± 316.55)	N/D	N/D	3394.51 _{7/7} (± 477.26)
α -Isosparteine	433.62 _{4/7} (± 159.12)	N/D	N/D	353.84 _{4/7} (± 117.25)
β -Isosparteine	N/D	N/D	193.33 _{4/7} (± 55.49)	N/D
Lupanine	N/D	450.81 _{7/7} (± 56.75)	1320.86 _{7/7} (± 664.48)	N/D
Sparteine	N/D	313.51 _{3/7} (± 184.74)	477.64 _{1/7}	N/D
Thermopsine	1006.42 _{7/7} (± 77.91)	N/D	N/D	661.75 _{7/7} (± 144.87)

Table S4.2 Concentrations ($\mu\text{g/g}$) of alkaloid species observed in *Lupinus bakeri* tissues. Subscripts denote the number of individuals that a given alkaloid was detected in out the total number of individuals sampled. Error estimates are $\pm 1\text{SE}$. N/D = Not detected.

	Pollen	
	Upper Loop	Kapuchion
Ammodendrine	N/D	N/D
Anagyrene	N/D	N/D
Aphyllidine	N/D	N/D
Argyrobine	N/D	N/D
5,6 dehydrolupanine	N/D	N/D
11,12 dehydrolupanine	N/D	N/D
5,6 dehydro- α -isolupanine	N/D	N/D
Dihydroxyaphyllidine	N/D	N/D
3-Hydroxy-lupanine	N/D	N/D
α -Isolupanine	N/D	N/D
α -Isosparteine	N/D	N/D
β -Isosparteine	N/D	N/D
Lupanine	N/D	N/D
Sparteine	N/D	N/D
Thermopsine	N/D	N/D
unknown	N/D	N/D

	Flower	
	Upper Loop	Kapuchion
Ammodendrine	13588.69 _{7/7} (± 2050.90)	8904.39 _{7/7} (± 1496.39)
Anagyrene	N/D	N/D
Aphyllidine	N/D	N/D
Argyrobine	N/D	N/D
5,6 dehydrolupanine	N/D	N/D
11,12 dehydrolupanine	N/D	N/D
5,6 dehydro- α -isolupanine	13373.24 _{1/7}	N/D
Dihydroxyaphyllidine	N/D	N/D
3-Hydroxy-lupanine	N/D	N/D
α -Isolupanine	N/D	N/D
α -Isosparteine	N/D	N/D

Table S4.2 (continued)

Table S4.2 (continued)

β -Isosparteine	N/D	N/D
Lupanine	3889.77 _{1/7}	N/D
Sparteine	N/D	N/D
Thermopsine	N/D	N/D
unknown	600.43 _{4/7} (± 321.05)	554.865 _{5/7} (± 122.06)

Leaf

	Upper Loop	Kapuchion
Ammodendrine	21301.07 _{7/7} (± 2640.04)	12832.91 _{7/7} (± 2074.93)
Anagyrine	N/D	N/D
Aphyllidine	N/D	N/D
Argyrobine	N/D	N/D
5,6 dehydrolupanine	N/D	N/D
11,12 dehydrolupanine	N/D	N/D
5,6 dehydro- α - isolupanine	3319.9 _{1/7}	N/D
Dihydroxyaphyllidine	N/D	N/D
3-Hydroxy-lupanine	N/D	N/D
α -Isolupanine	N/D	N/D
α -Isosparteine	N/D	N/D
β -Isosparteine	N/D	N/D
Lupanine	1539.39 _{1/7}	N/D
Sparteine	N/D	N/D
Thermopsine	N/D	N/D
unknown	917.28 _{5/7} (± 101.06)	2403.55 _{4/7} (± 192.88)

Stem

	Upper Loop	Kapuchion
Ammodendrine	13297.08 _{7/7} (± 2097.07)	8511.34 _{7/7} (± 1998.58)
Anagyrine	N/D	N/D
Aphyllidine	N/D	N/D
Argyrobine	N/D	N/D
5,6 dehydrolupanine	N/D	N/D
11,12 dehydrolupanine	N/D	N/D
5,6 dehydro- α - isolupanine	N/D	N/D
Dihydroxyaphyllidine	N/D	N/D

Table S4.2 (continued)

3-Hydroxy-lupanine	N/D	N/D
α -Isolupanine	N/D	N/D
α -Isosparteine	N/D	N/D
β -Isosparteine	N/D	N/D
Lupanine	N/D	N/D
Sparteine	N/D	N/D
Thermopsine	N/D	N/D
unknown	289.36 _{4/7} (± 29.44)	514.89 _{5/7} (± 99.31)

Table S4.3 Concentrations ($\mu\text{g/g}$) of alkaloid species observed in *Lupinus sulphureus* tissues. Subscripts denote the number of individuals that a given alkaloid was detected in out the total number of individuals sampled. Error estimates are $\pm 1\text{SE}$. N/D = Not detected.

	Pollen	Flower
Ammodendrine	N/D	N/D
Anagryne	1636.84 _{7/7} (± 1074.30)	11228.68 _{7/7} (± 2344.32)
Aphyllidine	N/D	N/D
Argyrolbine	N/D	N/D
5,6 dehydrolupanine	N/D	1363.61 _{7/7} (± 291.89)
11,12 dehydrolupanine	N/D	N/D
5,6 dehydro- α -isolupanine	N/D	N/D
Dihydroxyaphyllidine	N/D	N/D
3-Hydroxy-lupanine	N/D	N/D
α -Isolupanine	N/D	N/D
α -Isosparteine	N/D	N/D
β -Isosparteine	N/D	N/D
Lupanine	668.48 _{7/7} (± 306.98)	7116.86 _{7/7} (± 1809.99)
Sparteine	N/D	N/D
Thermopsine	N/D	N/D
unknown	N/D	N/D
	Leaf	Stem
Ammodendrine	N/D	N/D
Anagryne	3368.74 _{7/7} (± 716.36)	1571.20 _{7/7} (± 754.34)
Aphyllidine	N/D	N/D

Table S4.3 (continued)

Argyrolbine	N/D	N/D
5,6 dehydrolupanine	1658.27 ₇₇ (± 347.15)	2783.95 ₇₇ (± 474.03)
11,12 dehydrolupanine	N/D	N/D
5,6 dehydro- α - isolupanine	N/D	N/D
Dihydroxyaphyllidine	N/D	N/D
3-Hydroxy-lupanine	N/D	N/D
α -Isolupanine	N/D	N/D
α -Isosparteine	N/D	N/D
β -Isosparteine	N/D	N/D
Lupanine	10619.26 ₇₇ (± 1964.00)	3748.81 ₇₇ (± 749.60)
Sparteine	N/D	N/D
Thermopsine	N/D	N/D
unknown	N/D	N/D