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ARTICLE

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Consequences of pollen defense compounds for pollinators and antagonists in a pollen-rewarding plant

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Abstract

Plants produce an array of defensive compounds with toxic or deterrent effects on insect herbivores. Pollen can contain relatively high concentrations of such defense compounds, but the causes and consequences of this enigmatic phenomenon remain mostly unknown. These compounds could potentially protect pollen against antagonists but could also reduce flower attractiveness to pollinators. We combined field observations of the pollen-rewarding *Lupinus argenteus* with chemical analysis and laboratory assays to test three hypotheses for the presence of pollen defense compounds: (1) these compounds are the result of spillover from adjacent tissues, (2) they protect against pollen thieves, and (3) they act as antimicrobial compounds. We also tested whether pollen defense compounds affect pollinator behavior. We found a positive relationship between alkaloid concentrations in pollen and petals, supporting the idea that pollen defense compounds partly originate from spillover. However, pollen and petals exhibited quantitatively (but not qualitatively) distinct alkaloid profiles, suggesting that plants can adjust pollen alkaloid composition independently from that of adjacent tissues. We found no relationship between pollen alkaloid concentration and the abundance of pollen thieves in *Lupinus* flowers. However, pollen alkaloids were negatively associated with bacterial abundance. Finally, plants with more alkaloids in their pollen received more pollinator visits, but these visits were shorter, resulting in no change in the overall number of flowers visited. We propose that pollen defense compounds are partly the result of spillover from other tissues, while they also play an antimicrobial role. The absence of negative effects of these compounds on pollinator visitation likely allows their maintenance in pollen at relatively high concentrations. Taken together, our results suggest that pollen alkaloids affect and are mediated by the interplay of multiple interactions.

KEYWORDS
antagonists, bees, floral chemistry, *Lupinus*, plant–animal interactions, pollen, pollinators, secondary metabolites

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INTRODUCTION

Mutualistic interactions have traditionally been studied in a pairwise manner (Biere & Tack, 2013; Strauss & Irwin, 2004). This tradition perhaps derives from a classical view that mutualism involves intimate relationships between a small number of species (Thomson, 2003; Waser et al., 1996). Yet, most organisms are embedded in complex webs of interactions with multiple antagonistic and mutualistic partners (Polis, 1991; Waser et al., 1996). This wider community context can play a major role in shaping species interactions and might be key to fully understanding how mutualisms evolve (Strauss & Irwin, 2004; Strauss & Whittall, 2006).

For example, while most floral traits can be readily reconciled with the mutualistic interaction between flowering plants and their pollinators (Harder & Johnson, 2009), other floral traits are puzzling in this strictly mutualistic context. In particular, the seemingly paradoxical presence of defensive compounds in floral rewards—nectar and pollen—has intrigued pollination ecologists in recent decades (Adler, 2000; Stevenson, 2020; Stevenson et al., 2017). Defensive secondary metabolites in pollen and nectar have been shown to deter, harm, or even kill certain pollinators (Adler & Irwin, 2005; Arnold et al., 2014; Cane et al., 2020; Detzel & Wink, 1993; Gegear et al., 2007; Irwin & Adler, 2008; Jones & Agrawal, 2016; Tiedeken et al., 2016; Trunz et al., 2020), raising the question why plants would poison or repel their mutualists (Cane et al., 2020; Stevenson, 2020). Defensive compounds in plants are typically thought to confer protection against herbivores or pathogens, suggesting that antagonistic, rather than mutualistic processes, could be responsible for the presence of these compounds in floral rewards.

Pollination is frequently exploited by antagonists, which can have important impacts on the pollination process (Hargreaves et al., 2009; Irwin et al., 2003; Strauss & Whittall, 2006; Vannette, 2020). For example, florivores—herbivores that consume floral tissues—can damage flowers and thereby reduce plant fitness (McCall & Irwin, 2006). Moreover, the interaction between plants and pollinators is not completely harmonious, as pollinators and plants are often in a conflict of interest: pollinators benefit from collecting as much reward as possible with the least expenditure of effort, while plants benefit from efficient pollen dispersal while minimizing resources invested in reward production (van der Kooi et al., 2021; Westerkamp, 1996).

Secondary metabolites in nectar, despite potentially being a result of "spillover" from other tissues (Adler et al., 2006; Manson et al., 2012), have been variously found to mediate plant interactions with pollinators and microbes (Barlow et al., 2017; Johnson et al., 2006; Kessler et al., 2012; Kessler & Baldwin, 2007; Lokvam & Braddock, 1999; Stephenson, 1981; Wright et al., 2013). The functional significance of secondary metabolites in pollen, by contrast, remains largely unexplored, even though pollen often possesses higher concentrations of secondary metabolites than nectar—sometimes comparable to those of vegetative tissues (Cook et al., 2013; Palmer-Young et al., 2019; Rivest & Forrest, 2020).

We recently proposed three hypotheses to explain the presence of defensive secondary metabolites in pollen (Rivest & Forrest, 2020). First, these compounds might occur in pollen simply as a by-product of their production in other plant tissues. In this scenario, secondary metabolites "spill over" from adjacent plant tissues or are incidentally expressed in pollen, where they serve no adaptive function (see Adler, 2000; Manson et al., 2012). This "pleiotropy" hypothesis predicts that the concentration and profile of secondary metabolites in pollen should be correlated with those of other plant tissues (although the concentrations of secondary metabolites in pollen could be lower than those in other tissues). Second, pollen secondary metabolites might serve as protection against excessive pollen collection by pollen consumers. Unlike nectar, pollen is not only a reward for pollinators; its primary role is as a carrier of the plant's male gametes (Willmer, 2011). There might therefore be strong selection of plants to reduce pollen loss to consumption by pollinators and pollen thieves (Palmer-Young et al., 2019). Indeed, most of the pollen removed by bees is ingested or stored in specialized structures for transport to the nest, where it is generally inaccessible for pollination (Müller et al., 2006; Westerkamp, 1991). Moreover, some insects such as thrips and pollen beetles (and sometimes bees) consume pollen but play a negligible role in pollination, effectively acting as pollen thieves rather than pollinators (Hargreaves et al., 2009). Finally, pollen secondary metabolites might function as antimicrobials (compounds that kill or reduce the growth of microorganisms). Pollen can be colonized by a diverse array of bacteria and fungi (although not, as far as is known, by archaea) (Fürnkranz et al., 2012; Kim et al., 2018; Manirajan et al., 2018; McFrederick & Rehan, 2016). Although the role of these microbes in pollination is still unclear (Vannette, 2020), many of the secondary metabolites found in pollen are known to have antimicrobial properties, hinting at a potential antimicrobial function (Rivest & Forrest, 2020).

Few tests of these hypotheses have been conducted to date. Wang et al. (2019) found that bitter saponins in *Dipsacus* pollen reduce pollen collection by bumble bees, increasing pollen transfer in this nectar-rewarding plant (i.e., *Dipsacus* does not rely on pollen as a reward for pollinators). Consistent with the pleiotropy hypothesis, three studies found that alkaloid concentrations in pollen

were correlated (albeit weakly) with those in other tissues (Heiling et al., 2019; Kessler & Halitschke, 2009; Trunz et al., 2020). However, to our knowledge, no study has simultaneously tested all three hypotheses, limiting our ability to assess the role of multiple drivers in shaping the occurrence of pollen secondary metabolites. Moreover, while nectar secondary metabolites sometimes deter pollinators (Adler & Irwin, 2005; Gegear et al., 2007; Irwin & Adler, 2008; Jones & Agrawal, 2016), the cost of pollen-defensive compounds in terms of pollinator attraction remains mostly unknown (but see Muth et al. (2016) and Francis et al. (2019) for the deterrent effect of a vegetative tissue alkaloid on the bumble bee *Bombus impatiens*).

Here, we tested the ability of the three hypotheses described above: (1) the pleiotropy hypothesis; (2) the protection-against-pollen-collection hypothesis; and (3) the antimicrobial hypothesis; to explain the presence of defensive secondary metabolites in the pollen of a bee-pollinated plant, *Lupinus argenteus* (Fabaceae). We also (4) tested the effect of pollen secondary metabolites on the behavior of legitimate pollinators of *L. argenteus*. *Lupinus* flowers are nectarless and produce pollen as the only reward for pollinators (Heiling et al., 2021), which allowed us to avoid potentially confounding effects of nectar chemistry on pollinators and antagonists. Pollen from this genus contains quinolizidine alkaloids (Heiling et al., 2019), which are known to be toxic to insect herbivores and mammals when present in vegetative tissues (Wink, 1992); however, the effects of these compounds on pollen-feeding insects and microbes have not previously been examined.

METHODOLOGY

We investigated the relationship between *L. argenteus* pollen alkaloids and putative antagonists and pollinators using a combination of chemical analysis, field observations, and laboratory assays. To test (1) the pleiotropy hypothesis, we collected pollen and petal samples from *L. argenteus* individuals, analyzed their alkaloid content, and determined the relationship between pollen and petal alkaloid concentrations. We selected petals for this test because of their proximity and structural homology to the pollen-bearing anthers. To evaluate (2) the protection-against-pollen-collection hypothesis, we counted insect antagonists in *L. argenteus* flowers from the same individuals used for pollen and petal sample collection, and tested whether floral alkaloid concentrations predicted numbers of antagonists. To evaluate (3) the antimicrobial hypothesis, we extracted and cultured bacteria from pollen to test whether pollen alkaloids predicted an abundance of culturable bacteria, and we performed antimicrobial assays with bacteria isolated from pollen to directly test the antimicrobial properties of pollen alkaloids. Finally, we tested (4) the effect of pollen alkaloids on pollinator behavior by observing pollinator visitation to *L. argenteus*, and by performing behavioral assays with captive bumble bees. These methods are described in detail below.

Study system

Lupinus argenteus Pursh is a herbaceous perennial legume that is abundant in alpine and subalpine meadows in the Rocky Mountains. Mature individuals produce self-incompatible flowers on multiple racemes (Dunn, 1956; Gori, 1989). *Lupinus argenteus* is pollinated by large-bodied bees from the genera *Bombus*, *Osmia*, *Andrena*, and *Megachile*, which activate a sophisticated pump mechanism allowing the release of pollen from a keel petal, where the pollen is normally concealed (Dunn, 1956). Despite this putative pollen-protection mechanism, small insects such as pollen beetles and thrips are often found consuming pollen in *L. argenteus* flowers. These insects are unlikely to contribute to pollination as they cannot contact the stigmas of *Lupinus*; they are therefore considered pollen thieves (Dunn, 1956). In addition, thrips frequently damage the petals of *L. argenteus*, indicating that they also act as florivores. Finally, we have observed that pollen from *L. argenteus* is colonized by multiple species of bacteria (but less frequently by fungi; see next section).

Observations and sampling of *L. argenteus* interactors

We sampled *L. argenteus* in three populations (401, EL, and GT; see Appendix S1: Table S1) in and around the Rocky Mountain Biological Laboratory (RMBL), Colorado, USA. Four plots were sampled per population, two in 2021 and two in 2022. The plots were selected to encompass areas of high *L. argenteus* density (at least 10 individuals in a 5 m^2 radius). The plots were at least 10 m from one another. We sampled 62 individuals in 2021 and 67 in 2022, for a total of 129 individuals sampled across the 12 total plots. Within a plot, we included every individual with approximately 30 open flowers or more, as smaller individuals might not produce enough pollen for the alkaloid analysis (see below).

For each *L. argenteus* individual, we first conducted 4 h of pollinator observation separated into periods of 30 min and spread over 3 days. Up to 10 individual plants were observed simultaneously. Observers alternated plots

between each 30-min observation period. Because the three *L. argenteus* populations were located at different elevations and therefore bloomed at different times, only one population was observed at a time. During observations, we recorded the number of pollinator visits to each plant, and, for each pollinator visit, we recorded the identity of the pollinator (to the finest taxonomic resolution possible without capturing the insect, typically genus) and the number of flowers visited. To control for potential confounding effects, we recorded the number of open flowers per individual as well as the number of conspecific flowers in a 1 m radius of the focal individual.

After the 4 h of pollinator observations, we counted the numbers of thrips and pollen beetles per flower and collected pollen samples. Approximately 40 fresh-looking flowers were sampled per plant (fewer in individuals with fewer than 40 open flowers). Within an inflorescence, all open flowers that did not show signs of wilting were sampled. For each flower, we gently opened the wing petals and counted the number of thrips and pollen beetles.

On the same flowers, we then collected pollen in sterile microcentrifuge tubes by pressing the keel petals (activating the *Lupinus* pollen pump mechanism), using gloves and sterile equipment, for analysis in the laboratory. The pollen samples were kept cool before plating by storing them without preservative in a cooler in the field and then in a refrigerator at 4°C in the laboratory. Pollen samples were processed within 12 h of sample collection. We weighed the pollen and then detached bacteria from the pollen by vortexing each sample for 10 min in autoclaved phosphate-buffered saline. The samples were diluted (1:5 and 1:10) and plated on LB agar medium supplemented with cycloheximide (an antifungal), after which the bacteria were incubated at ambient temperature (see Burdon et al., 2018; Junker et al., 2014). The number of colony-forming units (referred to henceforth as "bacterial abundance") was counted 6 days after plating (shorter durations might miss slow-growing species; see Ambika Manirajan et al., 2016 for a similar duration). Similar abundances were obtained using AC agar and LB agar supplemented with sucrose (results not shown). Because few fungal colonies were observed from pollen samples plated on yeast agar supplemented with the antibacterial chloramphenicol, we did not analyze the abundance of fungi.

We collected specimens of thrips, pollen beetles, and four of the most abundant bacteria morphotypes for later identification. Thrips specimens were sent to the Canadian National Collection (CNC) of Insects, Arachnids and Nematodes for identification. We identified pollen beetle specimens using the keys of Legner (2003) and Zanetti (2014). A voucher specimen was deposited at the CNC. For bacteria, a portion of the 16S rRNA gene was amplified using primers 27F-1492R and sent for sequencing at Genome Québec (Montréal, Canada). We then identified the bacteria using Basic Local Alignment Search Tool (BLAST) searches against GenBank. At each site, we collected a voucher specimen of *L. argenteus*; specimens were subsequently deposited at the RMBL herbarium.

Alkaloid extraction and analysis

After the observations and sampling of *L. argenteus* interactors, we bagged the same *Lupinus* plants that were used for observations to increase the amount of pollen available for alkaloid analysis. Three days later, we randomly sampled three to six inflorescences (approximately 50–100 fresh-looking flowers), after which the samples were brought to the laboratory and separated into petal and pollen material. We then extracted alkaloids from one petal and one pollen sample per *L. argenteus* individual, for a total of 131 samples per tissue. We analyzed the alkaloid concentration of our samples by gas chromatography–flame ionization detection (GC-FID). We also analyzed two representative petal samples and two representative pollen samples from each population by gas chromatography–mass spectrometry (GC–MS) to help with compound identification by comparing mass spectra fragmentation patterns with those from the literature (Kinghorn & Balandrin, 1985; Wink et al., 1995). A more detailed methodology for the alkaloid extraction and analysis is presented in Appendix S1 (see also Lee et al., 2007 for similar methods).

Bumble bee behavior assays

We conducted a no-choice behavioral assay to test how bumble bee pollen-collection behavior was affected by *Lupinus* pollen alkaloids. We first installed wooden boxes filled with cotton (bumble bee domiciles) at two sites near the RMBL in June 2022 (see Appendix S1: Table S1 for coordinates). These boxes were used to attract nest-searching queens and thereby establish bumble bee colonies. Approximately 1 month after the boxes were installed, we collected the occupied boxes and brought them to the laboratory. The colonies (*Bombus appositus* Cresson and *B. rufocinctus* Cresson) were fed with a 30% sucrose solution and pollen (commercial honey-bee pollen mixed with doubly distilled water) prior to the assays. Each colony was connected to a 60 cm × 40 cm × 40 cm flight cage to which the bees were allowed free access for approximately 5 days prior to the experiment to allow them to familiarize themselves with the cages and learn to forage on artificial flowers. During training, pollen and nectar were provided in the flight cages using artificial

flowers. Nectar was provided from closed transparent Petri dishes, each with a hole in the center filled with a small cotton ball (to act as a nectar wick). Artificial flowers used to provide pollen were made of a 1 cm long white pipe-cleaner, to which the pollen was applied, glued in the center of a 6 cm transparent Petri dish (Appendix S1: Figure S1; see Russell & Papaj, 2016 for similar designs).

We conducted assays on 51 bees from seven colonies over 2 days. During each assay, one worker bee was introduced to the flight cage at a time. One artificial flower was placed at the center of the cage, containing 25 mg of either control pollen or pollen spiked with 2 mg/g of thermopsine, an alkaloid found in the pollen of some *L. argenteus* populations (see below). Bees were assigned alternately to the control or alkaloid treatments. This concentration of thermopsine was slightly higher than the average concentration of alkaloids in *L. argenteus* pollen across sites and years (1.37 mg/g) but was well within the range of observed concentrations (see Figure 1). We used ground honey-bee pollen for the experiment (Frontier Co-op, Norway, Iowa). Thermopsine was mixed with 95% ethanol before being incorporated into the pollen, while only 95% ethanol was added to the control pollen. Ethanol was allowed to evaporate for at least 30 min before the assays. Using 25 mg of pollen ensured that the bees would not empty the flowers before the end of the assays. The time spent on the flower and the number of visits were recorded for 5 min after the bees first contacted the artificial flower (bees were not allowed to return to the colony during assays). Bees that did not contact the flower after 3 min were excluded from the assay, and each bee was used for a single assay after which they were euthanized. Because colonies were collected from the field, some of the workers used in the assays might have had prior experience with real flowers (although we did not observe any lupine in the sites from which the colonies were collected).

Bacterial growth assays

The four common bacterial morphotypes from *L. argenteus* pollen selected for identification were identified as *Brevundimonas* sp., *Priestia* sp., *Peribacillus* sp., and

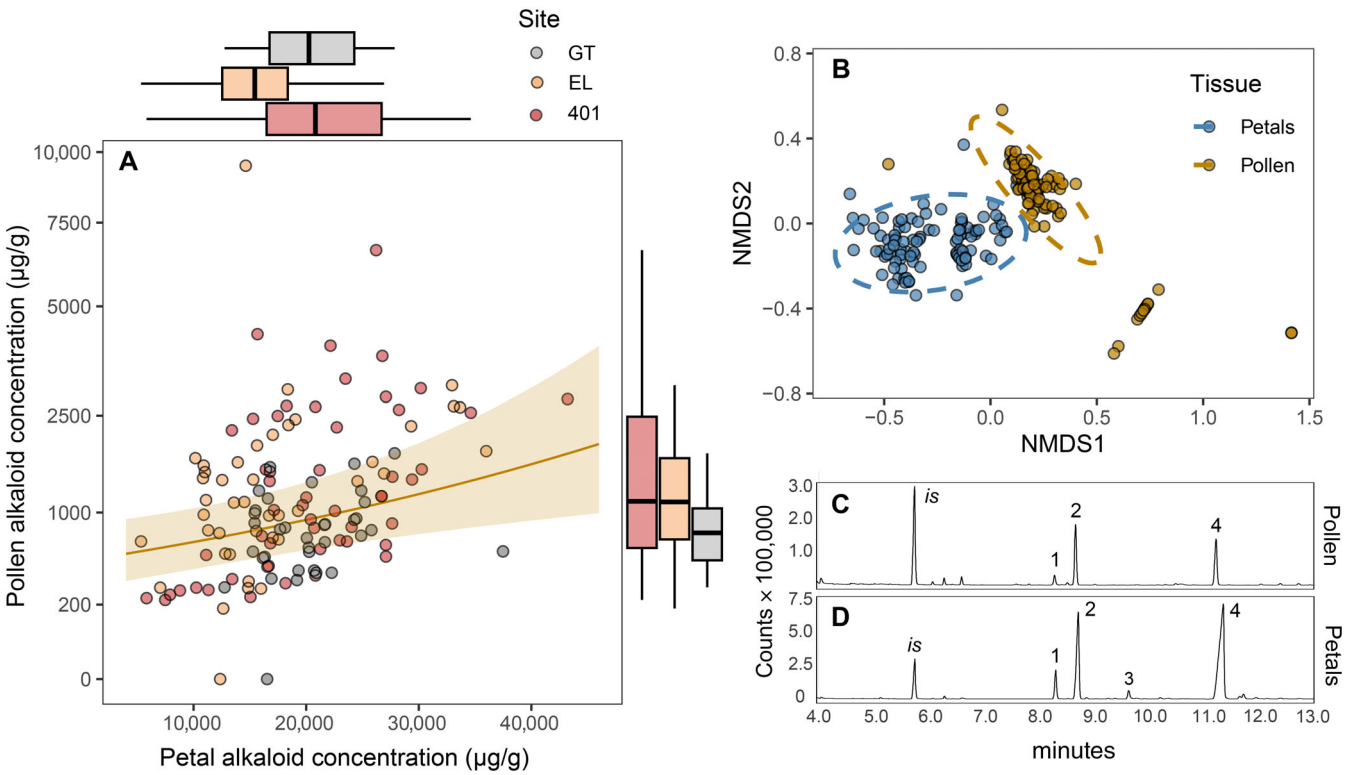


FIGURE 1 Concentration and composition of quinolizidine alkaloids in the pollen and petals of *Lupinus argenteus*. (A) Relationship between levels of alkaloids in petals and pollen, showing values for the three study sites. Note that pollen alkaloid concentration is shown on a square-root scale (the values were square-root transformed for analysis). (B) Nonmetric multidimensional scaling (NMDS2) ordination plot of the alkaloid profile in petals and pollen from the 401 and EL sites (number of alkaloids = 6, stress = 0.09). We analyzed individuals from the GT site separately because they belong to a different chemotype (see Appendix S1: Figure S2). Ellipses are drawn at a confidence level of 0.95. (C) GC-FID chromatogram representative of *L. argenteus* pollen. (D) GC-FID chromatogram representative of *L. argenteus* petals. Peak 1: 5,6-dehydro-α-isolupanine, peak 2: α-isolupanine, peak 3: 11,12-dehydrolupanine, peak 4: thermopsine, is: internal standard (caffeine).

Kocuria sp. (GenBank IDs: OR296832–OR296835). These bacteria are associated with plants (including Fabaceae), sometimes as epiphytes or endophytes, with some species found on flowers (De Meyer et al., 2015; Khanna et al., 2022; Semenzato et al., 2023; YanChun et al., 2011). Among these morphotypes, we randomly selected three morphotypes to test the effect of the *Lupinus* pollen alkaloid thermopsine on bacterial growth (*Brevundimonas* sp., *Priestia* sp., and *Peribacillus* sp.). The bacteria were grown overnight in LB broth at room temperature, and the concentration of each species was adjusted to a value of 0.1 OD_{600} (optical density at 600 nm on a spectrophotometer). For each morphotype, the bacteria were spread on five LB agar plates with sterile swabs. A 20 μL aliquot of a solution of thermopsine in dimethyl sulfoxide (DMSO) at concentrations of either 0, 2, 4, or 8 mg/mL was applied to sterile paper disks, and one disk of each concentration was placed on each agar plate (four disks per plate, 60 in total). DMSO was used as a solvent due to the low solubility of thermopsine in water. The 0 mg/mL thermopsine treatment was used as a negative control for the potential effect of DMSO on bacterial growth. The inhibition zones (zones without bacterial growth) around the disks were measured to the nearest 0.5 mm 48 h after the inoculation (see Junker et al., 2011).

Statistical analysis

We conducted all analyses in R version 4.1.1. (R Core Team, 2020). We checked for multicollinearity between explanatory variables in all our models using variation inflation factors (VIF) with the "check_collinearity" function from the *performance* package (Lüdecke et al., 2021) (all VIFs were lower than 2.5). We assessed model fits using the function "simulateResiduals" from the *DHARMa* package (Hartig, 2022).

Pleiotropy hypothesis

To test for a relationship between the total alkaloid concentrations in petals and in pollen, we used a generalized linear mixed model (GLMM), fitted with the *glmmTMB* package (Brooks et al., 2017) and "glmmTMB" functions, with a zero-inflated gamma error distribution and petal alkaloid concentration as the explanatory variable and year (a two-level categorical variable) as a covariate. Gamma distributions are well suited for continuous data confined to positive values and with skewed distributions (pollen alkaloid concentration was not normally distributed; Bolker, 2008). A zero-inflated distribution was used to handle the presence of zeros, which are not accommodated in standard gamma distribution models. Population (which was meant to be representative of the species more broadly) and plot (nested within a population) were included as random effects.

We compared the alkaloid profiles between pollen and petals using nonmetric multidimensional scaling (NMDS) with the Bray–Curtis dissimilarity index. We used the "metaMDS" function from the *vegan* package (Oksanen et al., 2020). We found two distinct chemotypes among our *L. argenteus* populations: one for the GT population and one for the EL and 401 populations. We therefore compared pollen and petal alkaloid profiles for each chemotype separately. For each chemotype, we removed a pollen sample for which no alkaloids were detected from the analysis.

Protection-against-pollen-collection hypothesis

To test for a relationship between pollen and petal alkaloid concentrations and the abundance of pollen thieves—the thrips and pollen beetles—in *L. argenteus* flowers, we again used GLMMs with zero-inflated gamma distributions. We used total pollen alkaloid concentration and total petal alkaloid concentration as explanatory variables as well as the number of flowers per plant, the density of conspecific flowers in the vicinity of the focal individual, and year as covariates. Two *L. argenteus* individuals for which we could not estimate the density of conspecific flowers (due to rodent damage on conspecific individuals during the sampling period) were removed from this analysis (sample size with removed individuals = 129). We square-root transformed the values of the number of flowers and pollen alkaloid concentration, here and in subsequent models, to reduce the impact of extreme values of this variable. Population and plot (nested within population) were included as random effects.

Antimicrobial hypothesis

We tested for a relationship between pollen alkaloid concentrations and bacterial abundance (as the number of colony-forming units per milligram of pollen) using the same model structure as for the analysis of pollen thieves above. Although petal alkaloids were not hypothesized to affect pollen-colonizing bacteria, we included petal alkaloid concentration as a covariate.

We tested the effect of the *L. argenteus* pollen alkaloid thermopsine on the growth of the isolated bacteria *Brevundimonas* sp., *Priestia* sp., and *Peribacillus* sp. using a GLMM with a zero-inflated gamma distribution. We used thermopsine concentration as the explanatory variable, and

bacterial taxon (only three taxa but meant to represent the pollen bacterial community more broadly) and plate (nested within bacterial taxon) as random effects (the replicates were the inhibition zones, with one of each treatment per plate).

Effect of pollen alkaloids on pollinator behavior

To test for a relationship between pollen alkaloid concentration and pollinator visitation, we used three measures of pollinator visitation summed across all pollinator taxa observed within the 4-h observation window: the number of pollinator visits, the average number of flowers visited per visit, and the total number of flowers visited per plant. For the model with the number of pollinator visits as the response variable, we first fitted the model with a Poisson distribution (to model count data) with the "glmer" function from the *lme4* package (Bates et al., 2015) and tested for overdispersion. Because we found overdispersion in the data (dispersion = 1.5), we used a negative binomial distribution (which controls for overdispersion in count data) with the "glmer.nb" function from the *lme4* package. We used a zero-inflated gamma distribution for the model of the total number of flowers visited and a gamma distribution for the number of flowers visited per visit. We removed plants that received no visits from the latter analysis because we did not have a measure of the number of flowers visited per visit for these individuals. We added the number of flowers per plant and the density of conspecific flowers in the vicinity of the focal individual as covariates (Two *L. argenteus* individuals for which we could not estimate the density of conspecific flowers were removed from this analysis). We incorporated petal alkaloid concentration as a covariate and used population and plot (nested within population) as random effects.

We tested whether the presence of the alkaloid thermopsine affected bumble bee behavior in laboratory assays by using GLMMs with a Poisson distribution for the number of visits to the artificial flowers and a gamma distribution for the time spent on the artificial flowers. In both models, we used treatment and species as fixed explanatory variables and colony as a random effect.

Direct and indirect interactions mediated by pollen secondary metabolites

Finally, we used piecewise structural equation modeling (SEM) to disentangle the direct and indirect ways in which pollen and petal alkaloid concentrations affect the interactions between *L. argenteus* and its mutualists and antagonists. This approach allowed us to better understand the community context in which *L. argenteus* exists, and to evaluate whether we omitted important variables from the individual models that could influence our conclusions (e.g., pollen-colonizing bacteria could influence pollinator visitation and vice versa). We incorporated the set of models presented above for all pollen interactors in the SEM (including the covariates and random effects used in the individual models) with the R package *piecewiseSEM* (Lefcheck, 2016). Only a single measure of pollinator visitation (number of flowers visited per visit) was incorporated into the model. We used the d-separation test (Shipley, 2009) to assess the fit of the SEM and identify missing paths in the model. We added the missing paths identified by the d-separation test to produce a final SEM. We then extracted coefficients from the final SEM and corrected *p*-values for multiple comparisons using the Benjamini–Hochberg procedure (Smith & Cribbie, 2013).

RESULTS

Alkaloid content of petals and pollen

We found 13 quinolizidine alkaloids as well as one unknown alkaloid across our petal and pollen samples of *L. argenteus* (Appendix S1: Table S2). Petals contained a higher alkaloid diversity and higher total alkaloid concentration than pollen (Figure 1). Moreover, the alkaloids found in pollen were always a subset of the alkaloids found in petals, that is, no quinolizidine alkaloids were unique to pollen (see Figure 1C,D). We observed considerable variation in alkaloid composition and concentration among and within populations (Figure 1A). Interestingly, pollen exhibited much higher variation in alkaloid concentration among individuals relative to petal alkaloids (coefficient of variation = 0.38 for petals and 0.95 for pollen; see also Figure 1A).

We found two distinct chemotypes across our *L. argenteus* populations: one for the GT population and one for the EL and 401 populations (Figure 1, Appendix S1: Figure S2). These two chemotypes exhibited little overlap in alkaloid composition (Appendix S1: Table S2). The observation of distinct chemotypes in nearby populations in the Colorado Rocky Mountains is consistent with previous studies on *L. argenteus* (Heiling et al., 2019; Wink & Carey, 1994).

Pleiotropy hypothesis

In support of the pleiotropy hypothesis, we found a positive relationship between petal and pollen alkaloid

concentrations in *L. argenteus* ($n = 131$, $Z = 3.06$, $p = 0.002$), although only a small portion of the variability in pollen alkaloid concentration was explained by the alkaloid concentration in petals (Figure 1A). However, despite the fact that pollen alkaloids were always a subset of the alkaloids found in petals, these tissues had quantitatively (but not qualitatively) distinct alkaloid profiles (i.e., alkaloids were present in different proportions in the different tissues), evidenced by the NMDS analysis (Figure 1B; Appendix S1: Figure S2). This difference was observed for both chemotypes present in our study populations, providing evidence against the pleiotropy hypothesis ($n = 195$ [number of individuals $\times 2$ tissues; one pollen sample for which we detected no alkaloids was removed from this analysis], $R^2 = 0.50$, $p = 0.001$ for the EL and 401 chemotypes, and $n = 65$ [one pollen sample for which we detected no alkaloids was removed from this analysis], $R^2 = 0.70$, $p = 0.001$ for the GT chemotype).

Protection-against-pollen-collection hypothesis

Pollen thieves were common in *L. argenteus* inflorescences: 88% of our sampled individual plants had thrips in their flowers, while pollen beetles were observed in 42% of individuals. Thrips sampled from *L. argenteus* flowers were identified as *Thrips vulgatissimus* Haliday (Thripidae) and *Odontothrips loti* (Haliday) (Thripidae), while the pollen beetles belonged to the species *Eusphalerum californicum* (Staphylinidae).

Despite the abundance of pollen thieves in *L. argenteus* inflorescences, we found no association between the concentration of alkaloids in pollen and the abundance of either pollen beetles ($n = 129$, $Z = -1.03$, $p = 0.30$) or thrips ($n = 129$, $Z = 0.012$, $p = 0.99$; Figure 2A,B). In contrast, we found a strong negative association between petal alkaloid concentration and thrips abundance ($n = 129$, $Z = -3.35$, $p < 0.001$), and a nonsignificant negative association between petal alkaloids and pollen beetle abundance ($n = 129$, $Z = -1.82$, $p = 0.070$; Figure 2A,B).

Antimicrobial hypothesis

We detected culturable bacteria in the pollen of 84% of *L. argenteus* individuals, although we likely missed low bacterial abundances that were under our detection threshold (which ranged from 1.5 to 111 colony-forming units per milligram pollen, depending on the mass of pollen sampled). We found a negative association between the concentration of alkaloids in pollen and the abundance of bacteria in pollen (measured as colony-forming units; $n = 129$, $Z = -3.02$, $p = 0.0026$; Figure 3A). Unsurprisingly, bacterial abundance in pollen was not associated with the concentration of alkaloids in petals ($n = 129$, $Z = 1.26$, $p = 0.21$).

In bacterial growth assays, naturally occurring concentrations of thermopsine did not reduce the growth of *Brevundimonas* sp., *Priestia* sp., and *Peribacillus* sp. isolated from *L. argenteus* pollen ($n = 60$, $Z = -1.08$, $p = 0.28$; Figure 3B). Inhibitory zones around the disks were either very small ($\leq$2.5 mm) or absent at concentrations of thermopsine ranging from 0 to 8 mg/mL.

Effect of pollen alkaloids on pollinator behavior

Most of the bees visiting *L. argenteus* belonged to the genus *Bombus*, but *Andrena*, *Osmia*, and *Megachile* were also frequent visitors. These bees activated the pump mechanism of *L. argenteus* flowers, indicating that they acted as legitimate pollinators (although we cannot infer pollination efficiency from these observations).

Surprisingly, we found a positive association between the concentration of alkaloids in pollen and the number of visits by bees (i.e., the total number of approaches to the plant that resulted in flower visitation), even when controlling for plant size and floral density of conspecifics in the surrounding 1 m ($n = 129$, $Z = 2.14$, $p = 0.032$; Figure 4A). In contrast, there was a negative association between pollen alkaloids and the mean number of flowers visited per visit ($n = 121$, $Z = -2.61$, $p = 0.009$; note that the sample size is lower in this analysis because we could not calculate the mean number of flowers visited per visit in individuals that received no pollinator visits; Figure 4B). However, this latter trend was largely driven by a few *Lupinus* individuals with very high pollen alkaloid concentrations: the relationship did not persist after the removal of the three highest values of pollen alkaloid concentration (>5000 μg/g) ($N = 118$, $Z = -1.581$, $p = 0.114$). The total number of flowers visited (i.e., the total number of flowers visited across all pollinator approaches) was unaffected by pollen alkaloid concentration ($n = 129$, $Z = 0.56$, $p = 0.57$). The results for the number of visits and total number of flowers visited were unchanged by the removal of the three extreme pollen concentration values.

In our laboratory assays, *Bombus* behavior was not significantly affected by the presence of thermopsine in naturally occurring concentrations (Figure 4C,D).

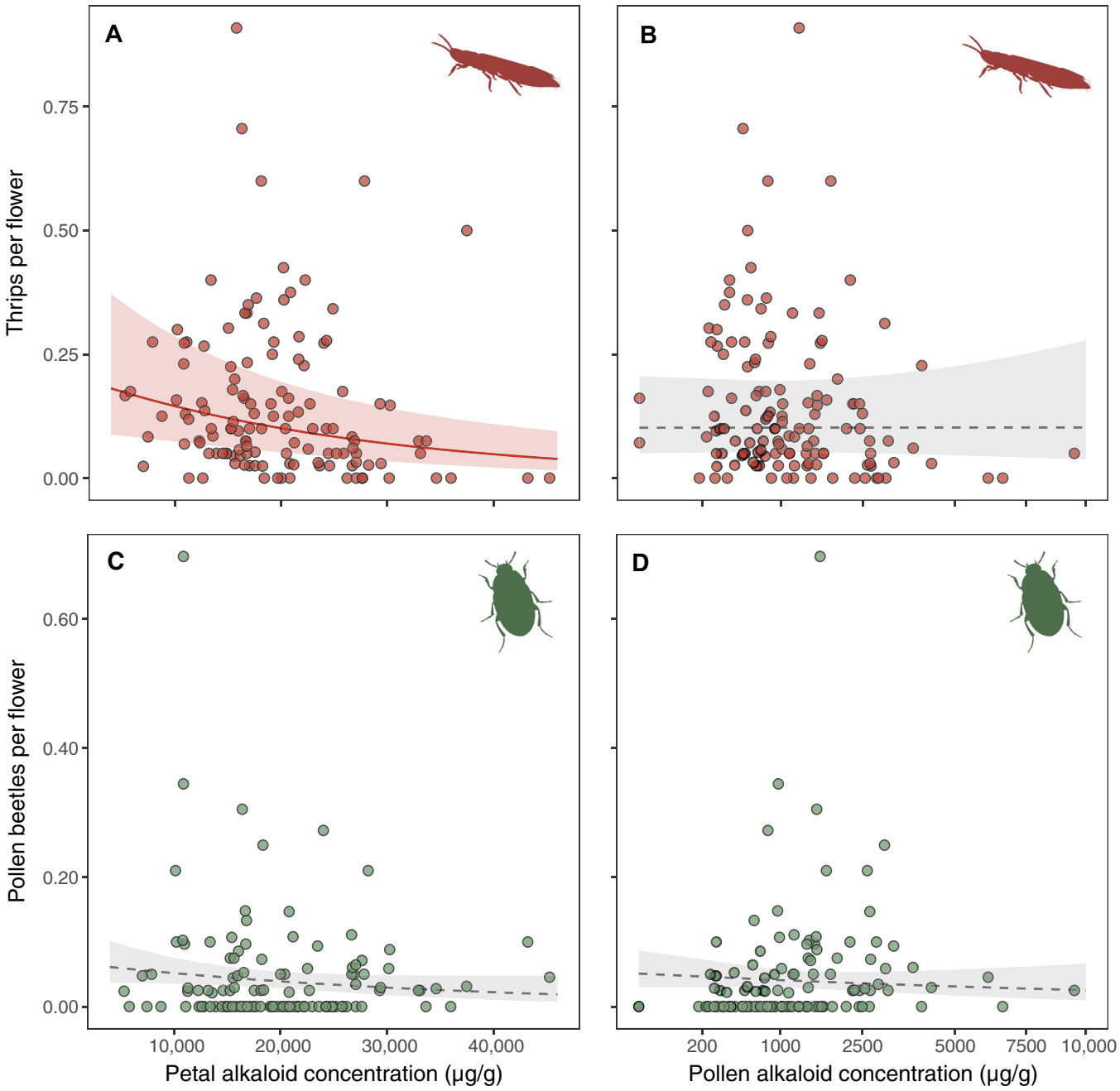


FIGURE 2 Relationships between *Lupinus argenteus* pollen (B, D) and petal (A, C) alkaloid concentrations (with pollen alkaloid concentrations on a square-root scale as in Figure 1A) and the abundance of two taxa of pollen thieves: (A, B) thrips, and (C, D) pollen beetles. Lines show predictions from the generalized linear mixed models, and solid colored lines show significant associations at alpha = 0.05. Illustrations created at BioRender.com.

Bumble bees tended to make fewer visits to alkaloid-supplemented pollen compared with control pollen, but this trend was not significant ($n = 51$: 27 for control pollen, 24 for alkaloid-supplemented pollen; $Z = -1.81$, $p = 0.070$; Figure 4C). There was no difference in time spent collecting pollen from artificial flowers ($n = 51$, $Z = -0.71$, $p = 0.48$; Figure 4D) between bumblebees exposed to alkaloid-supplemented or control pollen.

Direct and indirect interactions mediated by pollen secondary metabolites

The SEM analysis identified associations between *L. argenteus* interactors that were missing from the individual models (described in the sections above). Specifically, the abundance of pollen beetles in flowers was positively associated with the abundance

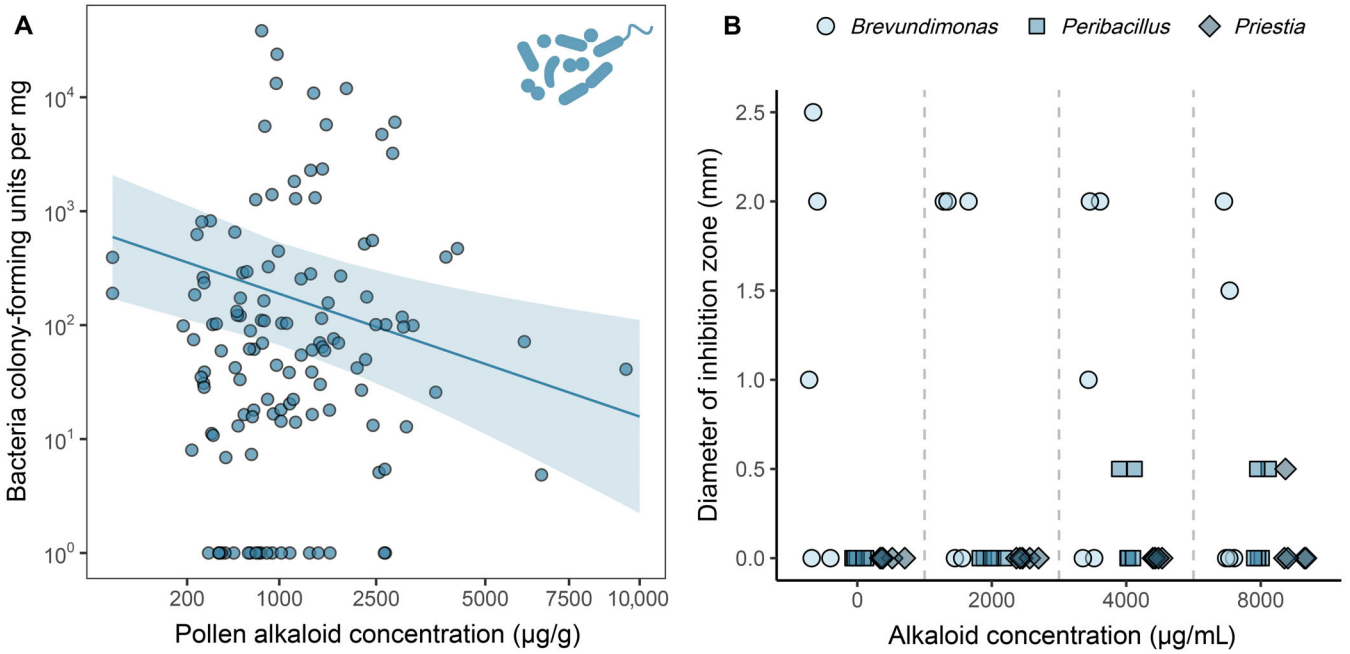


FIGURE 3 Antimicrobial effect of *Lupinus argenteus* pollen alkaloids. (A) Relationship between *L. argenteus* pollen alkaloid concentration (on a square-root scale) and the abundance of pollen-colonizing bacteria. (B) Inhibitory effect of different concentrations of the pollen alkaloid thermopsine on three species of bacteria isolated from *L. argenteus* pollen: *Brevundimonas* sp., *Priestia* sp., and *Peribacillus* sp. Illustrations created at BioRender.com.

of thrips and pollen-colonizing bacteria (Figure 5). However, incorporating these interactions in the SEM did not affect the conclusions from the individual models.

DISCUSSION

Defensive secondary metabolites are common in pollen and typically occur in considerably higher concentrations in this tissue relative to nectar (Cook et al., 2013; Palmer-Young et al., 2019; Rivest & Forrest, 2020; Trunz et al., 2020). Yet, the causes and consequences of this putatively defensive floral trait remain mostly unknown. We simultaneously tested three hypotheses to explain the presence of toxic secondary metabolites in pollen: (1) the pleiotropy hypothesis, (2) the protection-against-pollen-collection hypothesis, and (3) the antimicrobial hypothesis. We found some support for the pleiotropy and the antimicrobial hypotheses, but not for the protection-against-pollen-collection hypothesis. We also found that pollen alkaloids predict patterns of pollinator visitation but are unlikely to have a strong negative impact on pollination success. Taken together, our results suggest that pollen alkaloids affect and are mediated by the interplay of multiple antagonistic and mutualistic interactions.

Why are there defense compounds in pollen?

At face value, our results show mixed support for the pleiotropy hypothesis. Alkaloid concentrations in pollen increased with alkaloid concentrations in petals across *Lupinus* individuals, albeit weakly, suggesting that selection for alkaloid production in other tissues is partly responsible for their presence in pollen (Figure 1A). However, alkaloid profiles were quantitively distinct between pollen and petals, providing evidence that *Lupinus* can nevertheless adjust the composition of alkaloids in pollen independently from that of petals (Figure 1B). We propose that alkaloids in flowers are responsible for a baseline level of alkaloids in pollen via pleiotropy or spillover, but that *Lupinus* can partly adjust pollen alkaloid concentration from this baseline level. The same conclusion was recently reached by Trunz et al. (2020) who, like us, found a weak but significant correlation between alkaloids in pollen and corollas across Boraginaceae species. Interestingly, Trunz et al. (2020) also observed a higher rate of evolution of alkaloid concentration in pollen relative to corollas, showing that pollen alkaloids have some potential to evolve independently from other tissues. Our results are also consistent with those of Heiling et al. (2019), who found a correlation between alkaloids in pollen and some other plant tissues

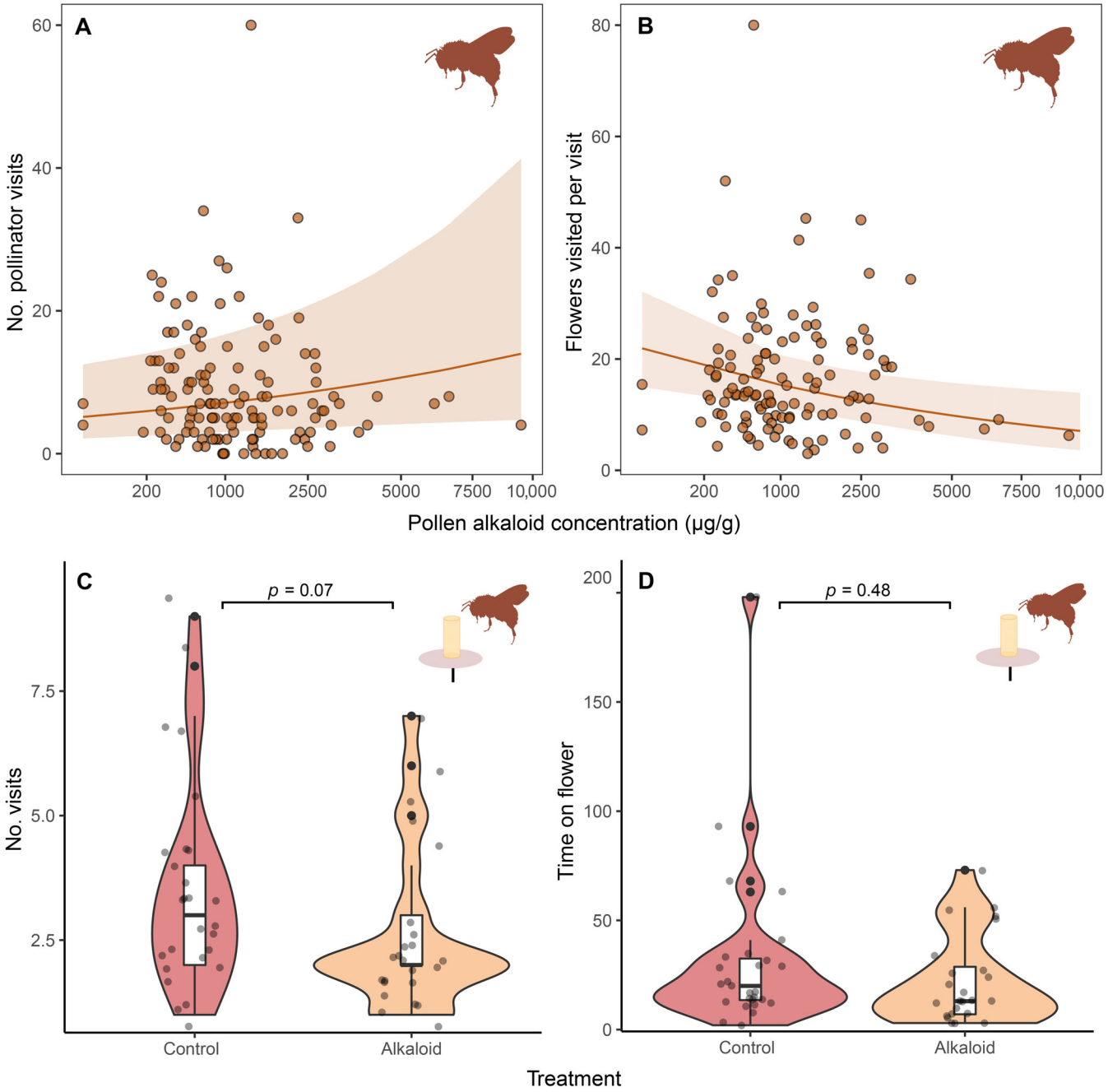


FIGURE 4 Relationships between *Lupinus argenteus* pollen alkaloid concentration (on a square-root scale) and two components of pollinator visitation in the field: (A) number of pollinator visits and (B) average number of flowers visited per visit. Effect of the pollen alkaloid thermopsine (2 mg/g) on two components of *Bombus* visitation to artificial flowers in the laboratory: (C) number of visits to artificial flowers and (D) time spent on flowers ($n = 27$ for Control, 24 for Alkaloid; see *Results* section for full statistical results). Illustrations created at BioRender.com.

in three *Lupinus* species, although the strength of the correlation varied between species and tissues. Similar to those in nectar (Adler et al., 2006, 2012; Manson et al., 2012), secondary metabolites in pollen thus appear to be physiologically constrained and to originate at least partly from nonadaptive mechanisms (Heiling et al., 2019; Kessler & Halitschke, 2009; Trunz et al., 2020) such as shared expression patterns between pollen and adjacent tissues or passive spillover from these tissues or the vascular system. This raises the question, however, of why pollen typically has higher concentrations of secondary metabolites than nectar.

As previously pointed out, the role of pollen as a carrier of the plant's male gametes might lead to the

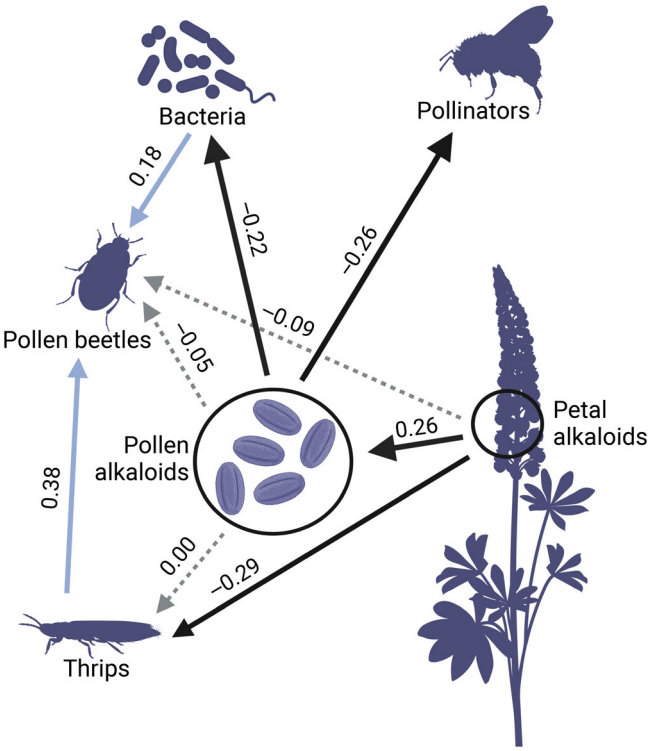


FIGURE 5 Path diagram showing the final structural equation model of the interactions between pollen and petal alkaloid concentration, and *Lupinus argenteus* interactors. Values of the standardized coefficients are given for each relationship, and significant relationships after correcting for multiple comparisons are denoted by solid black lines. Pale blue lines represent missing paths from the original model that were incorporated in the final model. See Appendix S1: Table S3 for the 95% confidence intervals around the coefficients. Illustrations created at BioRender.com.

selection of plants to protect their pollen against consumption (Heiling et al., 2019; Palmer-Young et al., 2019). Here, we found no support for the role of pollen secondary metabolites in protecting against two types of pollen thieves: thrips and pollen beetles (Figure 2). Instead, thrips abundance was negatively associated with alkaloids in petals (Figure 2B). Thrips consume both pollen and petals, so it is unsurprising that petal alkaloids appear to play a stronger role in deterring these antagonists, particularly given that alkaloids are more concentrated in petals than in pollen (Figure 1A). Pollen beetles from the genus *Eusphalerum*, which were not strongly deterred by either petal or pollen alkaloids in our study, are thought to consume exclusively pollen at the adult stage (Sayers et al., 2019). Although *E. californicum*, the species we found in *L. argenteus* flowers, is a generalist, these pollen consumers might have evolved tolerance to *Lupinus* alkaloids, as is suggested by the association of multiple *Eusphalerum* species with Fabaceae flowers (Zanetti, 2014). Overall, our results suggest that pollen thieves might often be tolerant of secondary metabolites in concentrations found in pollen, rendering such compounds an ineffective defense mechanism against pollen theft.

In addition to being consumed by an array of animals, pollen can be colonized by a high diversity of microorganisms (Fürnkranz et al., 2012; Kim et al., 2018; Manirajan et al., 2018; McFrederick & Rehan, 2016). We found that the abundance of bacteria in pollen was negatively associated with pollen alkaloid concentration in the field (Figure 3A). While this association could potentially originate from an indirect effect mediated by pollinators, which are important dispersers of flower microbes (Russell et al., 2019; Vannette et al., 2022; Zemenick et al., 2021), our SEM did not support such an indirect effect. It should be noted, however, that the cultivation-based approach that we used to determine bacterial abundance typically recovers only a small portion of the microbial diversity occurring on pollen (although Ambika Manirajan et al. (2016) found a good correspondence in the relative abundances of bacterial taxa recovered from cultivation-dependent and cultivation-independent approaches). Although we found a negative association between *L. argenteus* alkaloids and the overall abundance of culturable bacteria in pollen, it is possible that some bacteria are well adapted to the chemical defenses of *Lupinus* pollen, while other, perhaps less specialized, taxa could be inhibited by them. In support of this idea, we did not detect an effect of the *Lupinus* pollen alkaloid thermopsine on the three common bacteria isolated from *L. argenteus* pollen. These presumably common *Lupinus* colonizers appear to be highly tolerant of this *Lupinus* alkaloid.

While pollen alkaloids seem to exhibit some antimicrobial properties, it would be premature to conclude that microbes are the primary drivers of the evolution of pollen-defensive compounds. The roles of microbes in flowers are still unclear, and this is particularly true for those associated with pollen (Vannette, 2020). Pollen bacteria could reduce flower attractiveness to pollinators, as has been observed in some flower-colonizing microbes (Junker et al., 2014; Russell & Ashman, 2019; Vannette et al., 2012). In our study, however, the SEM analysis did not identify an effect of pollen bacteria on pollinator visitation. Bacteria might exert stronger selection on pollen defenses by directly affecting pollen function (Cullen et al., 2021). For example, yeasts colonizing nectar in the stigmatic cavities of *Asclepias* reduce pollen germination (Eisikowitch et al., 1990) while, in *Papaver*, nectar bacteria stimulate the germination of pollen grains that fall into nectar (Christensen et al., 2021). Pathogenic bacteria, fungi, and viruses can also colonize pollen and use stigmas as a doorway to infect plants (Card et al., 2007; Donati et al., 2018; Fetters et al., 2022; Huang et al., 2012),

potentially leading to strong selection for plants to kill these undesired hitchhikers if they reduce paternal fitness (for example by reducing siring success or progeny viability). More studies are clearly needed to assess the direct impact of pollen microbes on pollination. For example, future studies could examine the effects of pollen- and stigma-colonizing microbes on pollen germination and seed set. Moreover, studies investigating the role of pollen in carrying and transmitting plant pathogens could prove informative but are currently limited (see Card et al., 2007; Fetters et al., 2022).

Are pollen defense compounds ecologically costly to plants?

Plant secondary metabolites involved in defense against antagonists are often deterrents to herbivores. These compounds could deter pollinators as well when they occur in pollen (Francis et al., 2019; Muth et al., 2016). This might be especially true for plants that depend exclusively on pollen to attract pollinators. The presence of defense compounds in the pollen of *Lupinus* seems especially puzzling in this regard, given that plants from this genus have evolved relatively specialized pollination by pollen-collecting bees. We found that pollinators left alkaloid-rich *Lupinus* individuals after probing fewer flowers (Figure 4B), suggesting that bees can detect alkaloids in pollen and are deterred by it. However, this impact on pollinator behavior did not result in a lower number of flowers visited in total; in fact, plants with higher pollen alkaloid concentrations received slightly more pollinator visits (Figure 4A). Pollen alkaloids therefore seem to affect pollinator behavior, but not in a way that is likely to be costly to plant reproduction, at least for *L. argenteus*, as the effect of pollen secondary metabolites on pollinators might be highly species-specific and context-specific. It is also important to note that the negative association between pollen alkaloids and the number of flowers probed per pollinator visit was partly driven by a few *Lupinus* individuals with very high pollen alkaloid concentrations. This might explain why, in contrast with our field observations, we found only weak evidence for a deterrent effect of pollen alkaloids in our laboratory behavioral assays (Figure 4C,D). We only tested a single pollen alkaloid concentration (2 mg/g) and, as suggested by our field observations, pollen alkaloids might deter pollinators only at higher concentrations than the one we tested in the laboratory. Moreover, we only used a single compound (thermopsine), which might not have the same deterrent properties as the full blend of alkaloids observed in *Lupinus* pollen. Last, the relationship we observed between pollen alkaloid concentration and the number of flowers visited per bee visit could have originated from associations between pollen alkaloids and unmeasured plant traits affecting pollinator visitation rather than from a direct deterrent effect of pollen alkaloids. For example, some plants are confronted with a trade-off between the allocation of limited resources to defense and to traits responsible for pollinator attraction (Strauss et al., 2002), although we could not test whether such a trade-off played a role in the visitation patterns we observed.

The absence of a reproductive cost of pollen-defensive compounds in terms of the total number of flowers visited can help explain the paradoxical presence of alkaloids in *Lupinus* pollen. Concentrations in the range we observed could be maintained in pollen without bees imposing strong selection against them. That said, the few *Lupinus* individuals with the highest pollen alkaloid concentrations received both few visits and short visit durations, indicating that bees might select against very high concentrations of pollen alkaloids. In more typical concentrations, however, the effect of pollen alkaloids on pollinator behavior might even be beneficial to plants. Indeed, plants with more alkaloids in their pollen received the same total number of flower visits, but these visits were divided into shorter visitation bouts. This change in pollination pattern might reduce pollen loss to geitonogamy and promote cross-pollination. Plants have evolved multiple strategies to encourage pollinators to rapidly leave a plant and move to another one (Johnson & Nilsson, 1999; Kessler et al., 2012). Notably, nectar secondary metabolites have been shown in a few instances to serve such a function (Kessler et al., 2012; Kessler & Baldwin, 2007); pollen secondary metabolites may have a similar function in *Lupinus*. Future studies could directly test this hypothesis by tracking pollen dispersal from plants with various concentrations of pollen secondary metabolites, for example using a fluorescent dye (Adler & Irwin, 2006) or quantum dots (Minnaar & Anderson, 2019).

While our results provide potential explanations for the presence of defense compounds in pollen and help resolve the paradoxical presence of alkaloids in *Lupinus* pollen, *Lupinus* pollination is not typical of most angiosperms. Only ~ 20,000 species are exclusively pollen-rewarding (out of approximately 300,000 angiosperms in total; Willmer, 2011), and only a fraction of these species conceal their pollen like *Lupinus*. We should therefore be careful in generalizing our results to other plant species, as pollen secondary metabolites might occur for reasons other than those indicated by our studies with *L. argenteus*. For example, in contrast with our findings, Wang et al. (2019) found that bitter saponins in the pollen of nectar-rewarding *Dipsacus* reduce pollen collection by bumble bees,

suggesting that pollen secondary metabolites might sometimes protect against pollen collection in plants that offer nectar as the primary reward.

Pollen defenses in a community context

Taken together, our results suggest that the presence of defensive compounds in pollen is driven by multiple interactions, both direct and indirect, with both mutualists and antagonists. Our SEM analysis supports this interpretation (Figure 5). Floral traits involved in multiple interactions with both mutualists and antagonists, such as pollen defenses, might therefore be hard to understand without considering the complex community context in which they are embedded. Such floral traits, which have sometimes been considered paradoxical in the narrow context of the mutualistic interaction between plants and pollinators, appear less puzzling in this broader context. Indeed, we believe our findings can help explain why pollen is sometimes seemingly defended, even in pollen-rewarding plants: In *Lupinus*, bacteria and florivores (thrips, via the selection on correlated traits) could, both directly and indirectly, drive higher concentrations of pollen defense compounds, while pollinators are unlikely to select for reductions in defensive compounds, except when these compounds are extremely concentrated. Nevertheless, much remains to be understood about the ecological drivers of pollen defenses, especially how the impacts of different drivers, both mutualistic and antagonistic, vary within and among species. Studies are needed that directly test the effect of pollen defenses on plant fitness (for example by spiking pollen with secondary metabolites in the field), and that manipulate the abundance of multiple flower interactors. Such studies promise to improve our understanding of how mutualistic interactions are shaped by the broader community context in which they are embedded.

AUTHOR CONTRIBUTIONS

Design, statistical analysis, and initial writing were done by Sébastien Rivest with supervision from Jessica R. K. Forrest; Stephen T. Lee, Daniel Cook, and Sébastien Rivest carried out chemical analysis. All authors contributed to the writing and revisions.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT

Data and code (Rivest et al., 2024) are available in Figshare at https://doi.org/10.6084/m9.figshare.25251193.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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