The causes and consequences of pollen defence

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Abstract

Animal pollination represents one of the key innovations of the flowering plants, and constitutes an essential ecological service in most ecosystems. While pollinators are the main drivers of flower evolution, some floral traits are puzzling when viewed only in the context of this mutualistic interaction. In particular, the pollen of plants belonging to several families has spines or compounds with toxic effects on insects. Little is known about the causes and consequences of these enigmatic floral traits. Yet, pollen defences might play an important role in pollination given that pollen is the main source of food of the principal pollinators in most ecosystems: bees. My thesis investigates why plants sometimes have seemingly defended pollen and how these putative defences affect host-plant use by bees. Given the potential role of flower-colonizing microbes in pollination, I also investigate the potential for these microorganisms to influence flower evolution. I found that pollinators are unlikely to act as potential agents of selection on the concentration of defence compounds in the pollen of *Lupinus argenteus*. Rather, physiological spillover or pleiotropy from tissues highly defended against herbivores might be responsible for a baseline level of defence compounds in pollen, while such compounds could also mediate the interaction between plants and pollen-colonizing microbes. However, I did not find evidence that flower-colonizing microbes drive the evolution of floral traits in an experimental study. I also found that pollen chemical and mechanical defences likely restrict pollen-host use by Osmiini, a group of solitary bees exhibiting high interspecific variability in their pollen diet. Bees tolerated the defences of their pollen hosts, but were often harmed by the pollen defences of co-occuring plants exploited by other Osmiini species. This pattern provides a striking parallel with the evolution of host-use in herbivorous insects feeding on vegetative tissues, and suggests that pollen defences might play an important role in structuring plant–bee

interactions. Overall, my thesis contributes to our understanding of the causes of the presence of chemical defences in pollen and their consequences for the pollination mutualism.

Résumé

La pollinisation par les animaux représente une innovation majeure des plantes à fleurs, et constitue un service écologique essentiel à la plupart des écosystèmes. Bien que les pollinisateurs soient les agents principaux dans l'évolution des fleurs, certains traits floraux semblent mystérieux dans le contexte mutualiste de cette interaction. En particulier, le pollen de diverses familles de plantes possède des épines ou des composés toxiques pour les insectes. Nous ignorons toutefois quelles sont les causes et les conséquences de ces énigmatiques traits floraux. Pourtant, les défenses du pollen pourraient jouer un rôle important dans la pollinisation puisque le pollen constitue la source principale de nourriture des principaux pollinisateurs de la plupart des écosystèmes : les abeilles. Ma thèse examine pourquoi les plantes possèdent parfois des défenses putatives dans leur pollen, et comment ces défenses affectent le régime alimentaire des abeilles. Considérant le rôle potentiel des microbes colonisant les fleurs dans la pollinisation, j'examine aussi le potentiel de ces microorganismes à influencer l'évolution des fleurs. J'ai trouvé que les pollinisateurs ne jouent probablement pas un rôle considérable en tant qu'agents de sélection sur la concentration de composés de défense dans le pollen de *Lupinus argenteus*. Plutôt, un débordement physiologique ou une pléiotropie des tissues fortement défendus contre les herbivores pourraient être responsables d'un niveau de base de composés de défense dans le pollen, alors que ces composés pourraient aussi influencer l'interaction entre les plantes et les microbes colonisant le pollen. Toutefois, je n'ai pas trouvé d'évidence que les microbes des fleurs influencent l'évolution florale dans une étude expérimentale. J'ai aussi trouvé que les défenses mécaniques et chimiques du pollen restreignent l'utilisation en plantes hôtes par Osmiini, un groupe d'abeilles variant considérablement entre espèces dans leurs régimes alimentaires. Les abeilles toléraient les défenses du pollen de leurs plantes hôtes, alors qu'elles

étaient souvent affectées négativement par les défenses d'hôtes exploités par d'autres espèces d'Osmiini. Ce patron évolutif fourni in parallèle flagrant avec l'évolution du régime alimentaire des insectes herbivores, et suggère que les défenses du pollen pourraient jouer un rôle considérable dans la structure des interactions entre les plantes et les abeilles. Dans l'ensemble, ma thèse contribue à notre compréhension des causes de la présence de défenses chimiques dans le pollen des plantes et de leurs conséquences sur la pollinisation.

Statement of Contributions

The research ideas, written content, analyses, and figures in the following chapters of this thesis are my own, although co-authors provided guidance and advice that contributed significantly to the quality of the work.

Chapter 2 of this thesis is adapted from an article published in the peer-reviewed journal New Phytologist (Rivest and Forrest 2020). I performed the review of the literature necessary for this article and wrote a draft of the manuscript. Jessica Forrest helped develop ideas, provided comments on the writing, and added writing of her own to the final version of the article.

Chapter 3 is currently in review at the peer-reviewed journal Ecology to be published as a research article (Rivest, S., Lee, S. T., Cook, D., Forrest, JRK. 2023. Consequences of pollen defense compounds for pollinators and antagonists in a pollen-rewarding plant. In review at Ecology). I developed the ideas for this chapter. I performed field and laboratory work with help from Florence Jean. Analyses of pollen and petal chemistry were performed by Stephen Lee, Daniel Cook, and me. I performed the statistical analyses and wrote a draft of the manuscript. Jessica Forrest, Stephen Lee, and Daniel Cook provided comments and helped with the writing of the final version of the manuscript.

I developed the ideas for Chapter 4 (Rivest, S., Muralidhar, M., Forrest, JRK. 2023. Pollen chemical and mechanical defenses restrict host-plant use by bees) with the help of Jessica Forrest. I performed field and laboratory work with the help of Florence Jean and Erin Francispillai. Additionally, Lydia Wong and Jessica Forrest provided some of the bee nests used for larval diet manipulation. I performed the statistical analyses and wrote a draft of the manuscript. Madhupreeta Muralidhar helped with the construction of the phylogenetic tree used

in the manuscript. Jessica Forrest and Madhupreeta Muralidhar provided valuable comments on the writing.

I developed the ideas and conceived the design of the study for Chapter 5 (Rivest, S., Forrest, JRK. 2023. Do flower-colonizing bacteria influence floral evolution? A test with fast-cycling Brassica). I performed the laboratory work necessary for the chapter with the help of Lori Fernandez and Sarah Knoerr. I performed statistical analyses, and wrote a draft of the manuscript, on which Jessica Forrest provided comments and helped with the writing.

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Chapter 1: General introduction

Mutualistic interactions have traditionally been studied in a pairwise manner (Biere and Tack 2013). This tradition perhaps derives from the initial view that mutualism involves intimate relationships between organisms (Waser et al. 1996). For instance, one of the primary goals in the field of pollination ecology has been to understand how suites of floral traits are associated with pollination by certain types of pollinators (i.e., pollination syndromes) (Herrera 1996, Fenster et al. 2004, Rosas-Guerrero et al. 2014, Dellinger 2020). Yet, viewing floral traits as the result of purely mutualistic and intimate interactions between plants and pollinators fails to explain a substantial portion of the incredible floral diversity that can be witnessed around the globe (Adler 2000, Armbruster and Armbruster 2002, Strauss and Whittall 2006, Huang et al. 2012, Kessler et al. 2013). One striking example of this is the seemingly paradoxical presence of mechanical and chemical defences in the pollen of diverse plant families (Rivest and Forrest 2020): secondary metabolites with deterrent or toxic properties against insects are not only found in vegetative tissues, but also frequently occur in nectar and pollen (Stevenson et al. 2017, Palmer-Young et al. 2019, Rivest and Forrest 2020, Trunz et al. 2020), and many types of pollen exhibit conspicuous spines (Pope 1925, Tellería 2017, Konzmann et al. 2019). The concentration of secondary metabolites in pollen even sometimes matches that of vegetative tissues (Rivest and Forrest 2020). These putative pollen defences are puzzling in the context of pollination, in which attraction is considered key to the success of the interaction. However, pollen defence is not only interesting for the apparent paradox that it presents from the perspective of plant fitness; pollen is also the main source of food of the most important group of pollinators—bees. In other words, the interest in pollen defences not only lies in understanding why plants seem to poison their

pollinators, but also in understanding how pollen defences affect the structure of this mutualism. The motivation for this thesis is that answering these questions will contribute to our understanding of pollination.

1.1 Thesis outline

I begin by documenting the known occurrences of chemically defended pollen across plant taxa through a literature review, and by presenting hypotheses to explain their presence: the pleiotropy hypothesis, the protection-against-pollen-collection-hypothesis, and the antimicrobial hypothesis (Chapter 2). I also discuss potential impacts of pollen defences on plant-pollinator interactions.

In Chapter 3, I test the hypotheses proposed in Chapter 2 to explain the presence of pollen chemical defences using a combination of field observations, chemical analyses, and laboratory assays with the plant species *Lupinus argenteus* and its pollen interactors. Alkaloids toxic to insect herbivores have recently been found in the pollen of this species, but in concentrations that varies considerably among individuals, even within populations (Heiling et al. 2019). Populations of *L. argenteus* in the Colorado Rocky Mountains are colonized by a wide range of interactors: bees, pollen thieves and herbivores such as thrips and pollen beetles, and pollen-colonizing bacteria, which allowed me to test all three hypotheses that I proposed in Chapter 2 using this system.

In Chapter 4, I determine whether pollen chemical and mechanical defences restrict host-plant use by bees. Multiple researchers have been struck by the similarities in evolutionary patterns of food use between bees and herbivorous insects (Sipes and Tepedino 2005, Sedivy et al. 2008, 2013, Müller and Kuhlmann 2008). In herbivores, such patterns are thought to be mainly driven

by plant defences (Ehrlich and Raven 1964, Futuyma and Agrawal 2009), suggesting that pollen defences could similarly drive the evolution of pollen-host use by bees. I test this hypothesis by manipulating the presence of pollen mechanical and chemical defences in the diet of nine species of Osmiini, a group of solitary bees varying in their pollen diet.

In Chapter 5, I test whether flower colonizing microbes can influence floral evolution. Multiple studies have documented ecological impacts of flower microbes on pollination (Herrera et al. 2008, Herrera and Pozo 2010, De Vega and Herrera 2013, Helletsgruber et al. 2017, Vannette and Fukami 2018, Rering et al. 2018, Schaeffer et al. 2019), and as I discuss in Chapter 2 and 3, pollen chemical defences could have evolved as a defence against pollen colonizing microbes. In this chapter, I use an experimental evolution approach with fast cycling *Brassica rapa* to determine the separate and interactive effects of pollinators and bacteria on the evolution of multiple plant traits. In Chapter 6, I discuss my major results, synthesize these findings, and present important unanswered questions and propose future experiments to answer them.

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Chapter 2: Defence compounds in pollen: why do they occur and how do they affect the ecology and evolution of bees?

2.1 Abstract

Pollen plays two important roles in angiosperm reproduction, serving as a vehicle for the plant's male gametes, but also, in many species, as a lure for pollen-feeding animals. Despite being an important food source for many pollinators, pollen often contains compounds with known deterrent or toxic properties, as documented in a growing number of studies. Here we review these studies and discuss the role of pollen defensive compounds in the coevolutionary relationship between plants and bees, the preeminent consumers of pollen. Next, we evaluate three hypotheses that may explain the existence of defensive compounds in pollen. The pleiotropy hypothesis, which proposes that defensive compounds in pollen merely reflect physiological spillover from other plant tissues, is contradicted by evidence from several species. Although plants may experience selection to defend pollen against poor-quality pollinators, we also find only partial support for the protection-against-pollen-collection-hypothesis. Finally, pollen defences might protect pollen from colonisation by antagonistic microorganisms (antimicrobial hypothesis), although data to evaluate this idea are scarce. Further research on the effects of pollen defensive compounds on pollinators, pollen thieves, and pollen-colonising microbes will be needed to understand why many plants have chemically defended pollen, and the consequences of those defences for pollen consumers.

2.2 Introduction

Plants have developed a complex array of chemical defences in response to selection imposed by herbivores and pathogens (Moore *et al.*, 2014; Richards *et al.*, 2015). These defences influence interactions between plants and their natural enemies and are thought to have driven the evolution and diversification of insect herbivores (Ehrlich & Raven, 1964; Futuyma & Agrawal, 2009). In recent decades, a growing number of studies has documented the presence of compounds with deterrent or toxic properties in pollen and nectar (Stevenson *et al.* 2017 and references in Table 2-1), demonstrating that not only herbivores in the classical sense (i.e. consumers of vegetative tissue), but also pollinators, must confront plant defence compounds. In many instances, pollen and nectar appear to deter, harm or even kill pollinators (Adler, 2000; Cristina *et al.*, 2004; de Mesquita *et al.*, 2010; Junior *et al.*, 2011), suggesting that plant chemical defence could play an important ecological role in plant–pollinator interactions. However, how defence compounds affect pollinator ecology and evolution remains mostly unknown. A variety of hypotheses has been put forward to explain the existence of toxic or deterrent nectar (reviewed by Adler, 2000), but the possible reasons for the presence of defensive compounds in pollen – which differs fundamentally from nectar in its role in plant reproduction – have not previously been synthesised or evaluated.

Most plants use nectar as the primary reward for pollinators. Yet, many floral visitors also exploit flowers for pollen, which represents their principal source of protein and lipids. While nectar has evolved uniquely as a reward for pollinators, the principal function of pollen is to transport gametes between conspecific flowers. Therefore, although pollen can promote floral visitation by serving as reward, the collection of pollen by floral visitors for purposes of

consumption directly conflicts with pollen's primary function in plant reproduction (Willmer, 2011). Because the fate of pollen, unlike that of nectar, is directly linked to male reproductive success, there should be strong selection for plants to protect it from loss to consumption by floral visitors (Palmer-Young *et al.,* 2019).

While nectar generally contains lower concentrations of secondary metabolites than other plant tissues, concentrations of secondary metabolites in pollen can be similar to or even higher than those in leaves and other floral tissues (e.g. Kretschmar & Baumann, 1999; Bravo & Copaja, 2002). Consequently, pollen defence compounds have strong potential to affect plant–pollinator interactions. Yet, perhaps because of the dual role of pollen and the relative difficulty of manipulating its composition, most research concerning defence compounds in floral rewards has focused on nectar (Irwin *et al.*, 2014; Parachnowitsch & Manson, 2015; Stevenson *et al.*, 2017; but see Muth *et al.*, 2016 for a study of bee responses to a deterrent compound added to pollen). Furthermore, it has historically been assumed that pollen chemistry plays a trivial role in plant–pollinator interactions (Wcislo & Cane, 1996; Larkin *et al.*, 2008; Willmer, 2011). However, evidence from the phylogeny, behaviour, and diet breadth of bees (see section on 'Impacts of pollen defence on bee ecology and evolution') suggests that pollen defences, much more than nectar defences, could affect the evolution of this major clade of pollinators. Therefore, studying the causes and consequences of defence compounds in pollen could substantially advance our understanding of the processes shaping the evolution of pollination and pollinators.

Here, we first review what is known of the occurrence of pollen defence compounds across plant taxa. We then present evidence suggesting that pollen chemistry plays a more important role in

plant–pollinator interactions and bee evolution than assumed previously. Next, we present and evaluate three hypotheses to explain the presence of defence compounds in pollen of animal-pollinated plants. Finally, we suggest research directions that will advance our understanding of the causes and consequences of pollen defence.

2.3 Nature and occurrence of pollen defence

There have been several reports of compounds associated with plant defence against herbivores and pathogens occurring in the pollen of animal-pollinated plants. In this Viewpoint, we focus on secondary metabolites with known detrimental effects on animals. We exclude most volatile compounds that, when present in flowers, function primarily to attract pollinators from a distance, and will not necessarily be ingested by pollen consumers. However, it is important to note that many of those volatile compounds could also have defensive functions (Dobson & Bergström, 2000). Defence secondary metabolites have been observed in the pollen of at least 13 plant families and 23 genera (Table 2-1). These include widespread and locally abundant species that represent important dietary resources for some pollinator taxa.

The reported defence compounds in pollen are primarily alkaloids, but fatty acid derivatives, cyanogenic glycosides, flavonoids, iridoid glycosides, hydroxamic acids, and terpenoids have also been observed (Table 2-1). Palmer-Young *et al.* (2019) found that the pollen of every one of 28 species investigated, belonging to 21 different families, contained at least one class of secondary metabolites, mainly flavonoids, alkaloids, chlorogenic acids and terpenoids. Although this study was not restricted to metabolites possessing defensive properties, it illustrates the complex nature of pollen secondary chemistry and shows that pollen possesses a greater chemical diversity than nectar. Concentrations of defence compounds are generally at least an

order of magnitude higher in pollen than in nectar, but lower than in other floral and vegetative tissues (references in Table 2-1). However, there are several examples of pollen having the highest concentration of defence compounds among measured tissues within a species (Kretschmar & Baumann, 1999; Bravo & Copaja, 2002; Shoji *et al.*, 2005; Kempf *et al.*, 2010; Gosselin *et al.*, 2013).

Several studies have reported on pollen having toxic properties or preventing the development of bees (Cristina *et al.*, 2004; Praz *et al.*, 2008; de Mesquita *et al.*, 2010; Sedivy *et al.*, 2011; Junior *et al.*, 2011; de Melo *et al.*, 2013; Bukovinszky *et al.*, 2017; Vanderplanck *et al.*, 2018). However, despite the growing number of studies documenting the presence of defence compounds in pollen and the toxic properties of some pollens, few studies have made a direct link between the presence of defence compounds in pollen and a detrimental effect on pollinators. A negative impact of pollen defence compounds on bumblebees (*Bombus terrestris*) has been observed for the alkaloid d-lupanine (found in *Lupinus mutabilis* pollen), which decreases the size and number of brood at ecologically relevant concentrations (Arnold *et al.*, 2014). Similarly, naturally occurring concentrations of the alkaloid echimidine (found in *Echium* pollen) prevent the development of solitary bee larvae (Trunz *et al.*, 2017a). Sedivy *et al.* (2012) investigated the role of the glucoside ranunculin in the inability of several solitary bees to use *Ranunculu*s pollen as larval food. Through the addition of ranunculin to the pollen and nectar provisions of solitary bee larvae, they demonstrated that this compound was not present at sufficient concentrations in *Ranunculu*s pollen to affect larval development. However, they did not investigate the impact of protoanemonin, the volatile toxic derivative of ranunculin, which is the major scent compound in *Ranunculu*s pollen (Bergström *et al.*, 1995; Jürgen & Dötterl, 2004), so the real-world effects of *Ranunculus* pollen toxins remain unclear.

Considerably more studies have manipulated the presence of defence compounds in nectar, showing detrimental impacts at concentrations beyond those observed in nectar but similar to those seen in pollen (Detzel & Wink, 1993; Kevan & Ebert, 2005; Cook *et al.*, 2013; see also Elliot *et al.*, 2008), suggesting that consumption of a large quantity of pollen containing those compounds could affect pollinator health.

2.4 Impacts of pollen defence on bee ecology and evolution

The role of pollen secondary metabolites in the ecology and evolution of pollinators remains virtually unexplored (Parachnowitsch & Manson, 2015). However, many observations suggest that pollen composition could affect the evolution of bees. For reasons that remain unclear, a large proportion of the Earth's *c*. 20 000 species of bees specialise on a limited number of plant taxa for pollen, but not for nectar (Wcislo & Cane, 1996). This intriguing contrast in level of generalisation suggests that pollen is a more complex resource than nectar to acquire or assimilate. If host characteristics limit host use and host transitions in a lineage, closely related species should use more similar (and therefore often more closely related) hosts than do distantly related species, a phenomenon called phylogenetic conservatism (Sedivy *et al.*, 2008). Pollen usage by bees is characterised by strong phylogenetic conservatism (Sipes & Tepedino, 2005; Sedivy *et al.*, 2008), indicating that flower characteristics of some kind limit floral-host transitions in bees. What remains unknown is the relative contributions of the various floral characteristics (flower morphology and colour, scent, pollen shape and nutrient content) in bee evolution, and whether pollen secondary chemistry plays a role (Danforth *et al.*, 2013). The patterns of floral host use by bees share striking similarities to those seen in other phytophagous insects, such as the high proportion of dietary specialists and phylogenetic conservatism in host-

plant use (Ehrlich & Raven, 1964; Wcislo & Cane, 1996; Sedivy *et al.*, 2008). In phytophagous insects, those patterns are mainly attributed to plant chemical and physical defences (Ehrlich & Raven, 1964; Futuyma & Agrawal, 2009), suggesting that similar plant characteristics could underlie the evolution of floral host use in bees.

Bee larvae are not able to develop on every type of pollen, and bee species differ in which types of pollen their larvae can tolerate (Williams, 2003; Praz *et al.*, 2008; Sedivy *et al.*, 2011; Haider *et al.*, 2014; Bukovinszky *et al.*, 2017; Vanderplanck *et al.*, 2018). Praz *et al.* (2008) studied larvae of four specialist bee species, comparing their ability to develop on pollen from different plant taxa. Although *Ranunculus*, *Echium* and Asteraceae all act as host plants for some specialist bees, pollen from these taxa made poor diets for other bee species, resulting in high mortality rates for nonspecialists. Interestingly, *Ranunculus* and *Echium* pollen are known to contain defence metabolites (Bergström *et al.*, 1995; Kempf *et al.*, 2010), suggesting that pollen defence could be responsible for the observed mortality. Indeed, Trunz *et al.* (2017a) observed a detrimental effect of a defence metabolite from *Echium* pollen (echimidine) on bee larval development, supporting the role of defensive chemistry in making *Echium* pollen unsuitable for nonspecialists. More generally, the variation among bee species in their abilities to tolerate various pollen types (Sedivy *et al.*, 2011; McAulay, 2018), and the apparent role of pollen secondary chemistry in determining whether larvae can develop on a given pollen, strongly suggest that different pollinator species have evolved physiological adaptations to tolerate the secondary chemistry of their hosts. If defence compounds prevent most bees from exploiting defended pollen (as in *Echium*), those compounds are likely to limit bees to using only those pollen hosts for which they can tolerate the secondary chemistry. Similarly, floral-host transitions should occur more often between pollen types requiring few adaptations on the part of

the exploiter, resulting in more transitions between related or biochemically similar hosts (i.e. phylogenetic conservatism).

Defence compounds in pollen could also favour specialisation in bees by allowing the use of resources otherwise unexploited by other consumers. In antagonistic plant–herbivore interactions, plants defended with specific compounds are protected against most herbivores, offering a dietary niche initially free of competition to the species able to tolerate those compounds (Ehrlich & Raven, 1964; Becerra & Venable, 2009). Two striking examples of co-dependence between specialist bees and their chemically defended hosts illustrate how pollen defence could favour bee specialisation. First, *Andrena astragali* only collects pollen from two species of the neurotoxic alkaloid-producing genus *Toxicoscordion* (= *Zigadenus*), and *Toxicoscordion paniculatum* is exclusively visited by this species of bee (Cane, 2018). Second, *Aconitum* spp., which contain defensive alkaloids in their pollen (Gosselin *et al.*, 2013), are the only host plants of the only two known pollen-specialist *Bombus* species, *B. consobrinus* and *B. gerstaeckeri*, and the former is the only effective pollinator of *Aconitum* in Scandinavia (Thøstesen & Olesen, 1996). In these examples, it seems probable that the exclusive capacity of those pollinators to tolerate the toxicity of their host's pollen allows them to exploit an otherwise unused resource. These examples are atypical of most plant–pollinator interactions, which rarely exhibit reciprocal specialisation (Memmott *et al.*, 2004, Bascompte & Jordano, 2013, but see Blüthgen *et al.*, 2008). Instead, pollen-specialist bees more often specialise on plants that can be pollinated by numerous pollinators or other pollen vectors (e.g. *Helianthus* spp.; Hurd *et al.*, 1980, *Larrea tridentata*; Minckley *et al.*, 1999, *Salix* spp.; Fowler & Droege, 2019), whether or not those pollinators consume their (potentially defended) pollen. Nevertheless, by limiting pollen consumption by nonspecialists (which may still visit flowers for nectar, and hence

contribute to pollination), pollen defences could create an unexploited resource for the bee species that can tolerate them.

If pollen defence is the main driver of pollen specialisation, we should observe a higher proportion of specialist bees associated with better defended pollens. The only test of this hypothesis so far found a trend in this direction within the family Boraginaceae (Trunz *et al.*, 2017a): two plant genera hosting numerous specialist bees were characterised by high concentrations of alkaloids in the pollen. However, the association was not consistent across the 16 plant genera studied, and a regression controlling for plant relatedness showed a nonsignificant correlation between pollen alkaloid content and number of specialist bees. These findings suggest that pollen chemical defences are not the only drivers of specialisation in bees, but that they could play a role in this plant lineage. Associations between pollen secondary chemistry and numbers of pollen-specialist bees remain to be investigated for other plant taxa.

While specialist bees have to tolerate the characteristics of a few host plants, perhaps by gaining an ability to detoxify specific defensive compounds, generalists must be able to exploit a diverse array of plants that may vary considerably in their floral chemistry. Therefore, generalist bees should evolve mechanisms to avoid harm from a variety of defence compounds, each of which may only be encountered occasionally. Indeed, several pollen types frequently used by common generalist taxa contain defence compounds (e.g. *Echium*, *Delphinium*, *Lupinus*, *Papaver*, *Solanum*; Table 2-1). Pollen mixing by many species of generalist bees has been suggested as a strategy to obtain all the nutrients required for development while also mitigating the harmful properties of different types of pollen (Eckhardt *et al.*, 2014). This strategy could be particularly important for the evolution of diet generalisation by allowing the use of pollen that would be

detrimental if not mixed with other pollen types. For example, bumblebees are the principal pollinators of *Lupinus*, but d-lupanine is detrimental to bumblebee larval development at only 10% of its average concentration in the pollen of *L. mutabilis* (Arnold *et al.*, 2014). In this case, pollen mixing appears essential for bumblebees to be able to use this type of pollen. Similarly, echimidine can reduce honey bee larval survival in concentrations observed in the pollen of *Echium vulgare*. However, larval exposure to echimidine is reduced by the transformation of pollen into royal jelly by nurse honey bees, which tolerate higher concentrations of this compound (Lucchetti *et al.*, 2018). It is not clear to what extent defence metabolites in pollen are responsible for the evolution of pollen mixing and transformation in bees, but those behaviours appear to at least help generalist bees cope with the negative impacts of defence compounds.

Despite defence compounds in pollen being potentially detrimental for nonspecialist bees at high concentrations, they could offer protection against parasites and pathogens to species specialising on defended pollen or be beneficial to generalists when included in their diet at low concentrations. Several studies have investigated the effects of nectar secondary metabolites on bee pathogens, and in some cases, these compounds reduce pathogen loads (Manson *et al.*, 2010; Richardson *et al.*, 2015). Considering the higher concentration of secondary metabolites in pollen than in nectar, defended pollen could confer similar beneficial effects. For example, members of the bee genus *Osmia* that specialise on Asteraceae pollen are protected against brood parasitism by *Sapyga* wasps that attack other *Osmia* species: the wasps avoid and are unable to develop on Asteraceae pollen provisions, for reasons that remain unclear (Spear *et al.*, 2016). Asteraceae pollen also reduced bumblebee infection by the pathogen *Crithidia bombi* and honeybee infection by *Nosema ceranae* in a controlled experiment (Giacomini *et al.*, 2018). In addition, Palmer-Young *et al.* (2017) demonstrated that six of seven tested pollen and nectar

secondary metabolites increased antimicrobial peptide expression in honey bees, subsequently reducing infection by deformed wing virus.

Although few studies have directly addressed this possibility, the several indirect lines of evidence presented here suggest that pollen defence could affect the ecology and evolution of bees and their co-evolution with flowering plants. Investigating those effects could greatly improve our understanding of pollinator behaviour, ecology and evolution. Studying pollen defence could also be valuable in the context of agriculture and anthropogenic biodiversity loss (see Roger *et al.*, 2017 for an example of a study on the effect of anthropogenic changes on pollen nutrient availability for pollinators). Habitat simplification, biodiversity loss and species invasions modify the types of plants available for foraging pollinators, often resulting in decreased resource diversity, which is associated with higher pollinator mortality (Goulson *et al.*, 2015; Carvell *et al.*, 2017). Reduced floral diversity might increase pollinator exposure to high concentrations of toxic compounds from certain plant species, and introduced plants could expose pollinators to new compounds for which they have not evolved tolerance (Tiedeken *et al.*, 2016).

2.5 Hypotheses to explain the existence of pollen defence compounds

Although research on pollen defence compounds remains scarce, various authors have proposed hypotheses to explain their existence (Fig. 2-1). We group these hypotheses in two categories: the pleiotropy hypothesis and the defence against pollen collection hypothesis. In the context of pollen collection, we do not make a distinction between pollen thieves and pollinators with low pollen-transfer efficiency, given that these categories actually represent different points along a continuum, and the same predictions can be made in both cases. In light of the recent finding that

pollen is colonised by a complex microbiome (see the 'Antimicrobial hypothesis' section later), we also add a third (antimicrobial) hypothesis. We do not include pollen allelopathy among our hypotheses, because, to our knowledge, the secondary metabolites responsible for allelopathic effects on other plants (Murphy & Aarssen, 1989; Murphy, 1999; El-Ayeb *et al.*, 2009) occur only in wind-pollinated or agamospermous plants and do not affect pollen consumers (Murphy, 1999). In other words, although they may play a role in plant–plant competition, they are not known to be defence compounds.

2.5.1 Pleiotropy hypothesis

Secondary compounds in pollen could be a pleiotropic consequence of production of those compounds in other plant organs. According to this hypothesis, pollen defence is not adaptive in itself, but rather is influenced by the concentration of defence compounds in nearby tissues or vascular fluids. Therefore, the pleiotropy hypothesis predicts that the concentration and qualitative composition of defence compounds in pollen will be correlated with those of other plant organs. In support of this idea, Kessler & Halitschke (2009) observed that concentrations of chlorogenic acid and rutin (a flavonoid) were correlated between leaves and pollen across individuals of *Solanum peruvianum*. However, Heiling *et al.* (2019) found that alkaloid content in pollen of *Lupinus* spp. was generally not correlated with the alkaloid content of flowers and vegetative tissues. Defence compounds are typically observed in pollen in lower concentrations than in leaves or petals, but higher concentrations in pollen have been observed in some species (Bonora *et al.*, 1988; Kretschmar & Baumann, 1999; Shoji *et al.*, 2005). Although a tissue having low concentrations of defence compounds does not prove that their occurrence is due to pleiotropy, a tissue having higher concentrations of defence compounds than other tissues is

more consistent with an adaptive rather than a pleiotropic cause (although it is not a definitive proof on its own).

Furthermore, the qualitative composition of the secondary metabolites of pollen and anthers differs considerably from that of other plant organs, suggesting that plants are at least partially able to control the production or allocation of those compounds in anthers and pollen (Dobson *et al.*, 1996; Kessler & Halitschke, 2009; Li *et al.*, 2016; Palmer-Young *et al.*, 2017). Nevertheless, quantitative or qualitative differences between vegetative tissues or petals and pollen do not rule out the possibility that defence compounds in pollen are a pleiotropic consequence of their accumulation in adjacent structures like anthers, where they could have defensive functions against florivores. Few studies have compared the secondary chemistry of pollen and anthers. In the few cases that have been examined, defence compounds are generally present in higher concentrations in anthers than in pollen, but the proportional accumulation of some compounds differs between the two tissues (Detzel & Wink, 1993; Cook *et al.*, 2013). In addition, for *Senecio*, the chemical profile of pyrrolizidine alkaloids differs between the pollen coat and the inside of the pollen grain (Kempf *et al.*, 2010), demonstrating that plants are able to control the production and allocation of secondary metabolites even at this minute scale.

In some instances, the possibility that pollen defence compounds originate from leakage from adjacent structures can be ruled out. Indeed, the observation of pyrrolizidine alkaloids in the pollen of *Symphytum officinale* but not elsewhere in the stamens (Stegemann *et al.*, 2018) makes clear for this species that pollen defence metabolites are not a pleiotropic consequence of their presence in other tissues. Additionally, in *Atropa belladonna*, the gene responsible for the production of the alkaloid scopolamine is expressed exclusively in the root pericycle cells, the

tapetum (the layer of cell surrounding the pollen mother cells and responsible for the production of the pollen coat), and the pollen mother cells (Suzuki *et al.*, 1999), suggesting a specific role of this metabolite in pollen.

In summary, widespread chemical dissimilarities between pollen and other plant tissues suggest that pleiotropy is generally not a sufficient explanation for the occurrence of pollen defence compounds. While, in many instances, the pleiotropy hypothesis cannot be ruled out, examples of specific secondary metabolites occurring in pollen but not in adjacent tissues demonstrate that pollen defence compounds are probably adaptive in at least some species.

2.5.2 Protection-against-pollen-collection-hypothesis

Pollen is used by several clades of animals, varying considerably in their efficiency at transporting pollen. Pollen consumers that contribute little to pollination are called pollen thieves. Pollen theft can arise from a temporal mismatch between flower visitation and stigma receptivity, a morphological or behavioural mismatch preventing the pollen thief from contacting the stigma, or the deposition of an insignificant proportion of the removed pollen despite contact with a receptive stigma (Hargreaves *et al.*, 2009). Pollen thieves are found in many arthropod groups including thrips, mites, flies and beetles and also in slugs; but the most common pollen thieves are bees (Hargreaves *et al.*, 2009). The large amount of pollen needed to rear their offspring (Schlindwein *et al.*, 2005; Müller *et al.*, 2006) and the behavioural flexibility of many generalist bees (Westerkamp, 1991) could predispose bees as a group to pollen theft. Even when foraging on plant species for which they are considered legitimate floral visitors, bees foraging for pollen remove a substantial proportion of pollen from anthers compared to other pollinator taxa and nectar foragers of the same species, and deposit a lower proportion of this pollen

(Thomson, 2003; Westerkamp & Claßen-Bockhoff, 2007). Therefore, plants could benefit from discouraging or minimising pollen collection, even by legitimate pollinators. Several authors have suggested that the inability of specialist and generalist bees to use nonhost pollen could be due to plants protecting their pollen from consumption by floral visitors (Praz *et al.*, 2008; Sedivy *et al.*, 2011; Haider *et al.*, 2014; Lunau *et al.*, 2015). Plants have developed many morphological mechanisms that prevent overexploitation of pollen by pollen-eating insects (Harder & Thomson, 1989), including heteranthery (Vallejo-Marín *et al.*, 2009), poricidal anthers (Harder & Barclay, 1994), nototrobic flowers (Müller, 1996), and keel flowers (Westerkamp, 1997). Defence compounds in pollen could serve the same function.

There is only one direct test of this hypothesis so far. Wang *et al.* (2019) recently demonstrated that greater concentrations of toxic saponin in *Dipsacus* pollen reduced the frequency with which bumblebees groomed pollen from their bodies into their pollen baskets, a behavioural change that was linked to greater deposition of pollen grains on stigmas. This study offers direct support for the protection-against-pollen-collection-hypothesis, but it is unclear from this single example how generally the hypothesis applies among plants with chemically defended pollen.

The Wang *et al.* (2019) study raises the question whether, or how generally, pollinators detect and adjust their foraging behaviour in response to the presence of secondary metabolites in pollen. Unlike other pollen collectors, bees forage for pollen that, for the most part, they will feed to their larvae rather than consuming themselves. Social apid bees (*Apis* and *Bombus* spp.) are a partial exception, in that adult workers do consume pollen in addition to feeding it to their larvae (Winston, 1987; Ruedenauer *et al.*, 2016; McAulay & Forrest, 2019), but we do not know whether they typically assess pollen by taste (using antennae, legs or mouthparts; see review by

de Brito Sanchez, 2011) before collecting it. An experiment by Muth *et al.* (2016) showed that the addition of quinine to pollen of artificial flowers reduced visitation by bumblebees (*B. impatiens*), suggesting that bitter-tasting defence compounds in pollen can be detected and avoided by this species. However, this study used a quinine concentration (10% of pollen mass) at least 10 times higher than the maximum amount of defence compounds observed in any pollen; furthermore, quinine is not known to occur in pollen, so the ecological relevance of this finding could be questioned. Some pollens known to contain considerable amounts of defence compounds are readily collected by generalist bees, suggesting that bees are not deterred by natural concentrations of those compounds. For example, *Lupinus* spp., which only offer pollen as a reward, are primarily pollinated by large-bodied bees (Dunn, 1956), despite the fact that pollen of some *Lupinus* species can contain enough alkaloids to harm those same bees (Arnold *et al.*, 2014; Heiling *et al.*, 2019). Furthermore, bumblebees did not show a preference for apple over almond pollen in a behavioural experiment (Ruedenauer *et al.*, 2016), despite the known high concentration of amygdalin in pollen of the latter species. In short, it is unclear how often bumble bees avoid pollen containing high concentrations of defensive compounds, and we know even less about the ability of other pollinators to assess the quality of the pollen they collect for their young (e.g. solitary bees) or consume themselves (e.g. flower beetles), despite the fact that such abilities must influence whether bitter-tasting secondary metabolites can deter pollen collection by pollinators.

If pollinators are driving the evolution of pollen defence in plants, we can first predict that plants with freely accessible pollen should be more chemically protected against pollen collection than plants in which pollen is inaccessible to low-quality pollinators (such that additional chemical defences should be unnecessary). Sedivy *et al.* (2011) noted that plants with pollen known to be

unfavourable for bees all exhibit open flower morphology with accessible anthers. However, recent observations do not support this statement. First, a recent study by Trunz *et al.* (2017a) observed no association between pollen accessibility and pyrrolizidine alkaloid content in pollen. In fact, they observed that some species in which pollen is completely inaccessible to bees had high concentrations of pyrrolizidine alkaloids. In addition, Fabaceae flowers have pollen hidden inside keel petals where it is accessible only to pollinators that effectively pollinate the flowers (Dunn, 1956), but nevertheless some Fabaceae species contain sufficient defence compounds in their pollen to impair pollinator development (Arnold *et al.*, 2014; Heiling *et al.*, 2019), or have been shown to be poor pollen sources for bee larvae (Haider *et al.*, 2014). Second, in orchids, pollen is packaged in structures called pollinia, making it impossible for pollinators to exploit pollen from this plant family (Johnson & Edwards, 2000). Yet, pollinia of the orchid genus *Phalaenopsis* are rich in pyrrolizidine alkaloids (Frölich *et al.*, 2006). Either some plant taxa have evolved multiple, seemingly redundant lines of defence, or defence against pollen collection is an insufficient explanation for the existence of chemically defended pollen.

A second prediction originating from the hypothesis that pollinators are the drivers of pollen defence is that pollen should be less defended when offered as the primary reward to pollinators, while plants not using pollen as a reward should defend their pollen against theft. To test this prediction, Trunz *et al.* (2017b) compared the pollen saponin content between primarily bee- and bird-pollinated species of *Sinningia*, hypothesising that pollen from bird-pollinated flowers should be better defended against pollen theft because it is not offered as a reward. They found only partial support for the hypothesis; however, it is important to note that in their study system, bee-pollinated plants had nototribic flowers (with anthers located in the dorsal part of the corolla), a morphology considered to be a form of protection against pollen collection by bees

(see above). Although Trunz *et al.* (2017b) assumed that these bee-pollinated plants were using pollen as a primary attractant to lure bee pollinators, it is unclear whether this was actually the case. Consequently, it is unclear whether we should in fact expect bee-pollinated *Sinningia* species to have less defended pollen. Regardless, a considerable proportion of the plant genera known to contain defence compounds in their pollen are primarily pollinated by bees, even sometimes using pollen as the only reward (e.g. *Echium*, *Delphinium*, *Lupinus*, *Papaver*, *Solanum*). Therefore, although a conclusive, quantitative analysis remains to be conducted, there is little indication that plants that use pollen as a reward have less defended pollen.

Although more studies are clearly needed, the evidence available to date does not support a primary role of bees in driving pollen defence. However, the conclusions from these studies do not apply to smaller pollen thieves such as thrips and pollen beetles, which could access hidden pollen and promote pollen defence even in those species with pollen inaccessible to bees. For example, thrips and pollen beetles have frequently been observed in the keel of *Lupinus* flowers, in which the pollen is concealed, even in species with alkaloids present in their pollen (J. M. Heiling, personal communication). Even though thrips can act as pollinators in several plant species, effective thrips pollination is often associated with a 'thrips pollination syndrome' (Scott-Brown *et al.*, 2019), and thrips are likely less effective at transporting pollen to conspecific stigmas in flowers adapted to pollination by larger animals (e.g. Dunn, 1956; Roy, 2001; Raju & Rao, 2006). Thrips and pollen beetles can consume a substantial amount of pollen and inflict damage on other flower parts (Dunn, 1956; Kirk, 1987; Nilsson, 1987; Williams, 1987); they could therefore represent an important antagonistic pressure in the evolution of pollen traits.

2.5.3 Antimicrobial hypothesis

The pollen coat covering the outer layer of the pollen grain (the pollenkitt) is rich in lipids and carbohydrates (Heslop-Harrison & Heslop-Harrison, 1985; Pacini & Hesse, 2005), potentially creating an attractive habitat for microbial colonists. Indeed, recent investigations have demonstrated that the surface of pollen grains is colonised by a diverse community of microorganisms (Fürnkranz *et al.*, 2012; Ambika Manirajan *et al.*, 2016; Kim *et al.*, 2018; Manirajan *et al.*, 2018); however, the impact of this microbiota on plant fitness is unknown. In nectar, colonisation by bacteria and yeast affects carbohydrate, amino-acid and secondary metabolite composition (Herrera *et al.*, 2008; Peay *et al.*, 2012; Vannette & Fukami, 2016; Rering *et al.*, 2018), with negative or positive impacts on pollinator attraction (Vannette *et al.*, 2012; Schaeffer *et al.*, 2019). It is possible that pollen-colonising microorganisms affect pollen composition in similar ways. Microorganisms could also potentially reduce the germination rate or siring success of pollen grains, or could affect the physicochemical properties of the pollen coat necessary for pollen adhesion to pollinators and stigmas. The study that comes closest to testing this hypothesis concerns the germination of pollen in the yeast-infected nectar of *Asclepias syriaca*. In *Asclepias*, nectar in the stigmatic cavities is necessary for pollen germination, which is inhibited when nectar is contaminated with the yeast *Metschnikowia reukaufii* (Eisikowitch et al., 1990). Direct infection of pollen by plant pathogens might also impair male fitness. For example, Huang & Kokko (1998) and Marques *et al.* (2013) have demonstrated that the fungi *Sclerotinia sclerotiorum* and *Colletotrichum acutatum* could penetrate and infect the pollen of *Medicago sativa* and *Citrus sinensis*, respectively. However, as both studies were conducted *in vitro* in suspensions of distilled water, we do not yet know whether this phenomenon occurs in nature.

Many studies have reported antimicrobial properties of pollen (Basim *et al.*, 2006; Abouda *et al.*, 2011; Morais *et al.*, 2011), suggesting that plants have experienced selection to protect their pollen against microorganisms. Plant secondary metabolites can have multiple functions (Joosten & Van Veen, 2011), and many pollen secondary metabolites known to affect herbivorous animals also possess antimicrobial properties (e.g. protoanemonin, Mares 1987; morphine, Rosenberg & Renkonen 1985; pyrrolizidine alkaloids, Joosten & Van Veen 2011; caffeine, Nonthakaew *et al.* 2015; scopolamine, Özçelik *et al.* 2011; catalpol, Dellar *et al.* 1996). Therefore, it is possible that compounds with defence properties against antagonists in vegetative tissues could serve as antimicrobial agents in reproductive structures, potentially helping plants mediate interactions with antagonist microorganisms.

2.6 Future directions

A good starting point to understand the existence of pollen defence chemicals would be to assess the heritability and within-individual and among-individual variability in the composition and concentration of these compounds. Knowledge of heritability of pollen defence traits would be useful for understanding their potential to respond to selection, while knowing their degree of intra- and interindividual variability will inform on the potential for floral antagonists to exert selection on those traits.

Regarding the role of pollen consumers in driving the evolution of pollen defences, the emphasis so far has been on bees, owing to their ubiquity in pollination systems, their substantial pollen requirements for brood-rearing, and their efficiency at harvesting pollen. However, the presence of defence compounds in the pollen of flowers that possess other bee-excluding mechanisms suggests that more attention should be brought to smaller, less conspicuous types of pollen

antagonists, including microorganisms. Manipulative experiments, similar to those that have tested the effects of nectar secondary metabolites on pollinators and antagonists (Adler & Irwin, 2005; Jones & Agrawal, 2016; Barlow *et al.*, 2017), are needed to test the role of pollen defences in plant–antagonist interactions. However, manipulating pollen defence compounds in natural conditions will likely be challenging. A potential way to circumvent this problem would be to combine experimental pollen manipulation in controlled settings with field studies of the intraspecific correlation between concentrations of pollen defences and visitation rates by pollinators and antagonists. These field studies would need to be accompanied by knowledge of within-individual variation in pollen secondary chemistry: if plants show low within-individual variation, levels of pollen secondary compounds could be measured in a subset of a plant's flowers, while the remaining flowers could be used to quantify visitation rates. However, if there is high within-individual variability, understanding the effect of pollen secondary chemistry on floral visitors would require measuring chemical traits and visitation for the same flowers. This would likely only be feasible with plant species in which anthers dehisce over several days, or that produce abundant pollen. In addition, researchers would benefit from simultaneously testing the effects of defence compounds on all of a plant's pollen interactors (including mutualists and antagonists) while also measuring the effects of those interactors on pollination processes. Those studies would not only allow us to infer the causes of defence compounds in pollen, but also to more broadly understand the role of antagonistic processes in the evolution of pollination.

Understanding the consequences of pollen defence for the ecology and evolution of bees will require a combination of approaches. First, phylogenetic studies testing for a correlation between phylogenetic proximity of bee species and similarity of host pollen secondary chemistry (controlling for other floral-host traits) would allow for determination of the importance of

pollen defence in shaping floral host use by bees. Likewise, searching for similarities between phylogenetically distant hosts exploited by closely related specialist bee species could give insight into the flower characteristics responsible for specialisation in bees (see Vanderplanck *et al.*, 2017). Another approach to understand the importance of pollen defence in bee evolution would be to test if pollen defence compounds are responsible for the observed unsuitability of various types of pollen for bees. Those experiments could be performed with cavity-nesting bees, which are often used to assess pollen suitability for larval development. Experimental 'trap-nests' colonised by these bees can be used to obtain newly completed bee brood cells, which can be manipulated to modify the content of defence compounds and test their effects on larval development and survival (Williams, 2003; Sedivy *et al.*, 2012; Trunz *et al.*, 2017a).

Finally, the recent discovery of the complex pollen microbiota demands investigation of its role in pollination (Manirajan *et al.* 2018, McFrederick & Rehan 2019). Indirect evidence points toward a role of pollen-colonising microorganisms in pollination. Experiments testing the effect of those microorganisms on different components of pollination and testing the effect of pollen defence on the pollen microbial community promise to bring new insights into the challenges plants face in their sexual reproduction and the strategies they use to overcome them.

2.7 Conclusion

Until recently, pollen chemistry was thought to play a small role if any in dictating the patterns of pollen usage by pollinators (Wcislo & Cane, 1996; Larkin *et al.*, 2008, but see Vanderplanck *et al.*, 2014, 2017). Pollination ecologists have tended to focus on the macronutrient content of pollen when considering its role as a resource for pollinators (Roulston & Cane, 2000), and on the external morphology of pollen when considering its role as a package

for transporting male gametes to stigmas (Willmer, 2011). However, new evidence suggests that pollen defence compounds could be a significant component of plant defence, plant–pollinator interactions and pollinator evolution. A growing number of studies document the presence of secondary metabolites with toxic or deterrent properties in pollen, demonstrating that this phenomenon is widespread across plant taxa. The actual function of pollen defence compounds is not yet clear, but in at least some instances these substances appear to serve an adaptive role in protecting pollen from herbivores (potentially including inefficient pollinators) or pathogens. Virtually all mutualistic relationships are influenced to some extent by antagonistic processes (Bronstein, 2001), and understanding how antagonists shape the evolution and ecology of pollination could greatly increase our understanding of this vital ecological process.

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Table 2-1 Reports of pollen containing compounds with defensive properties against animals. We omitted reports of secondary metabolites in pollen that lack clearly defined defensive properties against animals. Volatile compounds are excluded except for protoanemonin; see text.

Family	Genus	Compounds	Biological effects on animals	References
Acanthaceae	*Acanthus*	2,4-dihydroxy-1,4-benzoxazin-3-one and 2-benzoxazolinone (hydroxamic acids)	Decrease aphid (*Rhopalosiphum padi)* performance (Barria et al. 1992).	Bravo & Copaja (2002)
Asteraceae Boraginaceae	*Senecio, Eupatorium* *Echium* *Symphytum* *Heliotropium* *Myosotis*	Pyrrolizidine alkaloids	Strong feeding deterrents for most herbivores; liver-toxic for vertebrates and mutagenic for insects (Hartmann 1999).	Kempf *et al.* (2010) Stegemann *et al.* (2018) Trunz *et al.* (2017a)
Caprifoliaceae	*Dipsacus*	Saponin	Antifeedant and toxic for insect herbivores (Faizal and Geelen 2013).	Wang *et al.* (2019)
Fabaceae	*Lupinus*	Lupanine (alkaloid)	Toxic and repellent to insects (Emrich 1991, Kordan et al. 2012).	Detzel & Wink (1993); Arnold *et al.* (2014)
Gesneriaceae	*Sinningia*	Saponins	Antifeedants and toxic for insect herbivores (Faizal and Geelen 2013).	Trunz *et al.* (2017b)
Orchidaceae	*Phalaenopsis*	Pyrrolizidine alkaloids	Strong feeding deterrents for most herbivores; liver-toxic for vertebrates and mutagenic for insects (Hartmann 1999).	Kempf *et al.* (2010)
Papaveraceae	*Papaver*	Morphine, thebaine (alkaloids)	Pronounced effects on the central nervous system (Wittstock and Gershenzon 2002).	Larkin *et al.* (2007)
Plantaginaceae	*Chelone*	Catalpol and aucubin (iridoid glycosides)	Deterrents to generalist and non-adapted specialist herbivores (Deane Bowers and Puttick 1988, Bernays and Chapman 2001, Pankoke et al. 2010).	Richardson *et al.* (2016)
Ranunculaceae	*Ranunculus* *Pulsatilla* *Aquilegia*	Protoanemonin[1] (fatty acid derivative)	Insecticidal activity against *Drosophila melanogaster* and *Tribolium castaneum* (Bhattacharyya et al. 1993). Poisonous for livestock (Nachman and Olsen 1983).	Bergström *et al.* (1995); Jürgen & Dötterl (2004)
	Delphinium	Norditerpene alkaloids	Toxic to herbivorous insects and livestock (Jennings et al. 1986, Pfister et al. 2002).	Cook *et al.* (2013)
	Aconitum	Aconitine-like alkaloids	Neurotoxic and cardiotoxic to mammals and insects (Ameri 1998).	Gosselin *et al.* (2013)

Rosaceae	*Prunus*	Amygdalin (cyanogenic glycoside)	Metabolised to release hydrogen cyanide (HCN), a deadly poison to most animals (Kevan and Ebert 2005).	London-Shafir *et al.* (2003); Kevan & Ebert (2005)
Rutaceae	*Citrus*	Caffeine (alkaloid)	Inhibits insect feeding and is pesticidal (Nathanson 1984).	Kretschmar & Baumann (1999)
Solanaceae	*Brugmansia*	Scopolamine and other alkaloids	Increase mortality and prolong development of larvae of the generalist noctuid moth *Spodoptera frugiperda* (Alves et al. 2007).	Detzel & Wink (1993)
	Nicotiana	Nicotine and nornicotine (alkaloids)	Repellant and toxic to several types of insects including bees (Levinson 1976, Detzel and Wink 1993).	Detzel & Wink (1993)
	Solanum	Chlorogenic acid (ester) and rutin (flavonoid)	Slows development of the specialist *Manduca sexta* (Stamp and Ang 1996).	Kessler & Halitschke (2009)

[1]Protoanemonin is the volatile toxic derivative of ranunculin.

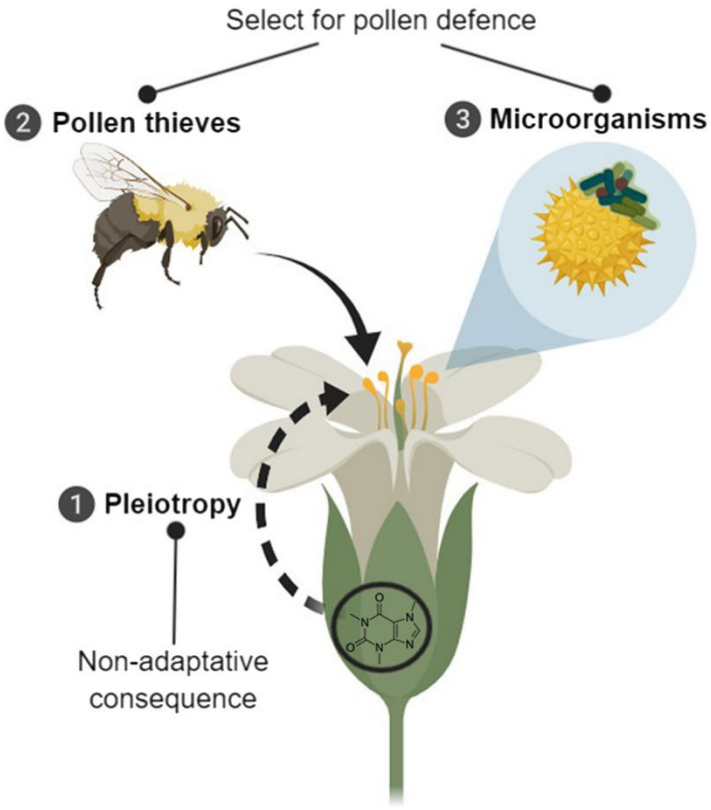

Figure 2-1 Potential causes of the presence of defence compounds in pollen. (i) Defence compounds in pollen could be a pleiotropic consequence of their accumulation in other plant tissues, (ii) they could serve as defence against loss of pollen to collection by floral visitors or (iii) they could function as antimicrobials against pollen-colonising microorganisms. Figure created with BioRender (https://biorender.io).

Chapter 3: Consequences of pollen defence compounds for pollinators and antagonists in a pollen-rewarding plant

3.1 Abstract

Plants produce an array of defensive compounds with toxic or deterrent effects on insect herbivores. Pollen can contain relatively high concentrations of such defence 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 defence compounds: 1) these compounds are the result of spillover from adjacent tissues, 2) they protect against pollen thieves, and 3) they act as antimicrobials. We also tested whether pollen defence compounds affect pollinator behaviour. We found a positive relationship between alkaloid concentrations in pollen and petals, supporting the idea that pollen defence 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 defence compounds are partly the result of spillover from other tissues, while they also play a role as antimicrobials. 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.

3.2 Introduction

Most floral traits can be easily understood in the context of the mutualistic interaction between plants and their pollinators, in which pollinators select on flowers' attractiveness and efficiency at dispensing and receiving pollen (Harder and Johnson 2009). For example, large, conspicuously coloured and fragrant flowers have repeatedly been shown to be favoured by pollinators (Fenster et al. 2004, Raguso 2008, Harder and Johnson 2009). Yet, 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 et al. 2017, Stevenson 2020). Defensive secondary metabolites in pollen and nectar have been shown to deter, harm, or even kill certain pollinators (Detzel and Wink 1993, Adler and Irwin 2005, Gegear et al. 2007, Irwin and Adler 2008, Arnold et al. 2014, Tiedeken et al. 2016, Jones and Agrawal 2016, Trunz et al. 2020, Cane et al. 2020), raising the question why plants would poison or repel their mutualists (Stevenson 2020, Cane et al. 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 that can have important impacts on the pollination process (Irwin et al. 2003, Strauss and Whittall 2006, Hargreaves et al. 2009, Vannette 2020). For example, florivores—herbivores that consume floral tissues—can damage flowers and thereby reduce plant fitness (McCall and 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 many rewards as possible with the least expenditure of effort, while flowers benefit from efficient pollen dispersal while minimizing energy invested in reward production (Westerkamp 1996, van der Kooi et al. 2021). For these reasons, the pollination mutualism is sometimes described as mutual exploitation (Westerkamp 1996, van der Kooi et al. 2021).

Recently, several studies have examined the causes of the presence of secondary metabolites in nectar. These compounds have been variously found to "filter" pollinators (i.e., exclude less-effective pollinators) (Stephenson 1981, Johnson et al. 2006, Barlow et al. 2017), manipulate pollinator behaviour (Kessler and Baldwin 2007, Kessler et al. 2012, Wright et al. 2013), and reduce microbial growth (Lokvam and Braddock 1999). A few studies have also pointed to a potential role of pleiotropy, or "leakage" from other plant tissues, in explaining the presence of secondary metabolites in nectar (Adler et al. 2006, Manson et al. 2012). The functional significance of secondary metabolites in pollen, by contrast, remains largely unexplored. Yet, 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 and Forrest 2020). This suggests that pollen secondary metabolites might play a more important role in plant–pollinator interactions, as pollinators can be exposed to higher concentrations of potentially toxic or deterrent compounds via pollen relative to nectar.

We recently proposed three hypotheses to explain the presence of defensive secondary metabolites in pollen (Rivest and 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 on 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 (scopae or corbiculae), where it is generally inaccessible for pollination (Westerkamp 1991, Müller et al. 2006). Moreover, some insects like 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. Pollen can be colonized by a diverse array of bacteria and fungi (archaea are not known from pollen) (Fürnkranz et al. 2012, McFrederick and Rehan 2016, Kim et al. 2018, Manirajan et al. 2018). 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 and 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).

In support of the pleiotropy hypothesis, three studies found that alkaloid concentrations in pollen were correlated (albeit weakly) with those in other tissues (Kessler and Halitschke 2009, Heiling et al. 2019, 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 and Irwin 2005, Gegear et al. 2007, Irwin and Adler 2008, Jones and Agrawal 2016), the cost of defensive compounds in pollen in terms of attracting legitimate pollinators 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*. We also (4) tested the effect of pollen secondary metabolites on the behaviour of legitimate pollinators of *L. argenteus*. *Lupinus* flowers are nectarless and only produce pollen as a 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.

3.3 Methodology

We investigated the relationship between *Lupinus argenteus* pollen alkaloids and putative antagonists and pollinators using a combination of chemical analysis, field observations, and laboratory assays. To answer (1) the pleiotropy hypothesis, we collected pollen and petal samples from *L. argenteus* individuals, analyzed their alkaloid content, and tested the correlation between pollen and petal alkaloid concentrations. 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 answer (3) the antimicrobial hypothesis, we extracted and cultured bacteria from pollen to test whether pollen alkaloids predicted 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 behaviour by observing pollinator visitation to *L. argenteus*, and by performing behavioural assays with captive bumble bees. These methods are described in detail below.

3.3.1 Study system

Lupinus argenteus Pursh is a herbaceous perennial that is abundant in alpine and subalpine meadows in the Rocky Mountains. Mature individuals produce self-incompatible, papilionaceous 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 like pollen beetles and thrips are often found consuming pollen in *L. argenteus* flowers. These insects are unlikely to contribute to pollination since 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 Methodology).

3.3.2 Observations and sampling of *L. argenteus* interactors

We sampled *Lupinus argenteus* in three populations (401, EL, and GT; see Table 3-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 lupine 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 four hours of pollinator observation separated in periods of 30 minutes and spread over three days. Up to 10 individuals were observed simultaneously. Observers alternated plots between each 30 minute 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 the number of flowers visited per visit. After the 4 hours of pollinator observations, we counted the numbers of thrips and pollen beetles

per flower. Approximately 40 fresh-looking flowers were randomly sampled per plant (fewer in individuals with fewer than 40 open flowers). For each flower, we gently opened the wing petals and counted the numbers of thrips and pollen beetles. On the same flowers, we then collected pollen by pressing the keel petals (activating the *Lupinus* pollen pump mechanism), using gloves and sterile equipment. The pollen samples were kept cool before plating. We weighed the pollen, and then separated bacteria from pollen by vortexing each sample for 10 minutes 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. The number of colony-forming units (referred to henceforth as "bacterial abundance") was counted 6 days after plating. 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 antibiotic chloramphenicol, we did not analyze the abundance of fungi. 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.

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 of Insects, Arachnids and Nematodes (CNC) for identification. We identified pollen beetle specimens using the keys of Legner (2003) and Zanetti (2014). 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.

3.3.3 Alkaloid extraction and analysis

After the observations and sampling of *L. argenteus* interactors, we bagged the *Lupinus* plants 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 lab 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 analysed 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 (Wink et al. 1995, Kinghorn and Balandrin 1984). More detailed methodology for the alkaloid extraction and analysis is presented in the Supporting information (see also Lee et al. 2007 for similar methods).

3.3.4 Bumblebee behaviour assays

We conducted a no-choice behavioural assay to test how bumble bee pollen-collection behaviour was affected by *Lupinus* pollen alkaloids. We first installed wooden bee boxes filled with cotton at two sites near the RMBL in June (See Table 3-S1 for coordinates). These boxes were used to attract nest-searching queens and thereby obtain established bumble bee colonies. Approximately one month after the boxes were installed, we collected the occupied boxes and brought them to the lab. The colonies (of *Bombus appositus* Cresson and *B. rufocinctus* Cresson) were fed with a 30% sucrose solution and pollen (commercial honey-bee pollen mixed with dd

water) prior to the assays. Each colony was connected to a 60 cm x 40 cm x 40 cm flight cage to which the bees were allowed free access for approximately five 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 with a hole in the centre 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 centre of a 6 cm transparent petri dish (Fig. 3-S1; see Russell and Papaj 2016 for similar designs).

We conducted assays on 51 bees from 7 colonies over two days. During each assay, one worker bee was introduced to the flight cage at a time. One artificial flower was placed at the centre 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 Fig. 3-1). We used ground honey-bee pollen for the experiment. Thermopsine was mixed with 95% ethanol before being incorporated in the pollen, while only the 95% ethanol was added to the control pollen. Ethanol was allowed to evaporate for at least 30 minutes 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 minutes 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 minutes 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).

3.3.5 Bacterial growth assays

The four common morphotypes from *L. argenteus* pollen selected for identification were identified as *Brevundimonas sp*., *Priestia sp*., *Peribacillus sp*., and *Kocuria sp*. (GenBank IDs: OR296832-OR296835). These bacteria are associated with plants (including Fabaceae), sometimes epiphytes or endophytes, with some species found on flowers (YanChun et al. 2011, De Meyer et al. 2015, Khanna et al. 2022, Semenzato et al. 2023). 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}. For each species, 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 mg/ml, 2 mg/ml, 4 mg/ml, or 8 mg/ml were applied to sterile paper disks, and one disk of each concentration was placed on each agar plate (4 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 presence of inhibition zones (zones without bacterial growth) around the disks was assessed 48 h after the inoculation.

3.3.6 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 correlation between the total alkaloid concentration in petals and in pollen, we used a zero-inflated gamma distribution (pollen alkaloid concentration was not normally distributed) with petal alkaloid concentration as the explanatory variable. Plot nested within population was included as a random effect. We square-root-transformed the values of pollen alkaloid concentration to reduce the impact of extreme values of this variable.

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.

Protection-against-pollen-collection hypothesis

To test for a relationship between pollen and petal alkaloid concentrations and the abundance of thrips and pollen beetles in *L. argenteus* flowers, we used GLMMs with zero-inflated gamma distributions with the function glmmTMB from the R package glmmTMB (Brooks et al. 2017). We used total pollen alkaloid concentration and total petal alkaloid concentration as explanatory variables and the number of flowers per plant, the density of conspecific flowers in the vicinity

of the focal individual, and year (a two-level categorical variable) as covariates. Plot nested within population was included as a random effect. We square-root-transformed the values of number of flowers and pollen alkaloid concentration to reduce the impact of extreme values of these variables.

Antimicrobial hypothesis

We tested for a relationship between pollen alkaloid concentrations and the number of colony-forming units per mg of pollen using the same model structure as for the analysis of pollen thieves above.

We tested the effect of the *L. argenteus* pollen alkaloid thermopsine on 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 plate nested within bacterial taxon as a random effect.

Effect of pollen alkaloids on pollinator behaviour

To test for a relationship between pollen alkaloid concentration and pollinator visitation, we used three measures of pollinator visitation summed across all pollinator taxa: 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 used a negative binomial distribution with the glmer.nb function from the lme4 package (Bates et al. 2015). 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. We incorporated petal alkaloid concentration as a covariate and used the same random effect structure as for the models for thrips, pollen beetles, and bacteria, presented above.

We tested whether the presence of the alkaloid thermopsine affected bumble bee behaviour 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 (using the function glmmTMB). 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 modelling (SEM) to disentangle the direct and indirect ways in which pollen and petal alkaloid concentrations affect the interactions between *L. argenteus* and pollen interactors. This approach allowed us to better understand the community context in which *L. argenteus* interacts with its pollinators and antagonists, 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 in the model. We used the test of d-separation (Shipley 2009) to assess the fit of the SEM, which allows one to identify missing paths in the model. We added the missing paths identified by the test of d-separation 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 and Cribbie 2013).

3.4 Results

3.4.1 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*. Petals contained a higher alkaloid diversity and higher total alkaloid concentration than pollen. Moreover, the alkaloids found in pollen were always a subset of the alkaloids found in petals—i.e., no quinolizidine alkaloids were unique to pollen (see Fig. 3-1C, D). The average alkaloid concentration across sites and years was 19,661 ± 7,208 μg/g (mean ± sd) for petals and 1,372 ± 1,310 μg/g for pollen. We observed considerable variation in alkaloid composition and concentration among and within populations (Fig. 3-1A). 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 Fig. 3-1A).

We found two distinct chemotypes across our *L. argenteus* populations: one for the GT population and one for the EL and 401 populations. These two chemotypes exhibited very little overlap in alkaloid composition (Table 3-S2). The observation of distinct chemotypes in nearby populations in the Colorado Rocky Mountains is consistent with previous studies on *L. argenteus* (Wink and Carey 1994, Heiling et al. 2019).

3.4.2 Pleiotropy hypothesis

In support of the pleiotropy hypothesis, we found a positive correlation between petal and pollen alkaloid concentrations in *L. argenteus* ($n = 129$, $Z = 3.31$, $P < 0.001$), although only a small

portion of the variability in pollen alkaloid concentration was explained by the alkaloid concentration in petals (Fig. 3-1A, Fig. 3-5). 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, evidenced by the NMDS analysis (Fig. 3-1B, Fig. 3-S2). This difference was observed for both chemotypes present in our study populations, providing evidence against the pleiotropy hypothesis ($n = 97$, $R^2 = 0.50$, $P = 0.001$ for the EL and 401 chemotype, and $n = 34$, $R^2 = 0.70$, $P = 0.001$ for the GT chemotype).

3.4.3 Protection-against-pollen-collection hypothesis

Pollen thieves were common in *L. argenteus* inflorescences: 88% of our sampled individuals 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 ($n = 129$, $Z = -1.029$, $P = 0.30$) or thrips ($n = 129$, $Z = 0.012$, $P = 0.99$) (Fig. 3-2A, 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 non-significant negative association between petal alkaloids and pollen beetle abundance ($n = 129$, $Z = -1.82$, $P = 0.070$) (Fig. 3-2A, B).

3.4.4 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 (approximately 1

colony-forming unit per mg, but the exact threshold varied between samples because of variation in the exact 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) ($n = 129$, $Z = -3.02$, $P = 0.0026$) (Fig. 3-3A). 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 ($n = 60$, $Z = -1.08$, $P = 0.28$) (Fig. 3-3B). Inhibitory zones around the disks were either very small or absent at concentrations of thermopsine ranging from 0 to 8 mg/ml.

3.4.5 Effect of pollen alkaloids on pollinator behaviour

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).

We found a positive association between the concentration of alkaloids in pollen and the number of visits by bees, even when controlling for plant size and floral density of conspecifics in the surrounding 1 m ($n = 129$, $Z = 2.14$, $P = 0.032$) (Fig. 3-4A). In contrast, there was a negative association between pollen alkaloids and the mean number of flowers visited per visit ($n = 129$, $Z = -2.61$, $P = 0.009$) (Fig. 3-4B). The total number of flowers visited was unaffected by pollen alkaloid concentration ($n = 129$, $Z = 0.56$, $P = 0.57$).

In our laboratory assays, *Bombus* behaviour was not significantly affected by the presence of thermopsine in naturally occurring concentrations. Bumble bees tended to make fewer visits to alkaloid-supplemented pollen compared to control pollen, but this trend was not significant ($n = 51$, $Z = -1.81$, $P = 0.070$) (Fig. 3-3C). There was no difference in time spent collecting pollen from artificial flowers ($n = 51$, $Z = -0.71$, $P = 0.48$) (Fig. 3-3D) between bumblebees exposed to alkaloid-supplemented or control pollen.

3.4.6 Direct and indirect interactions mediated by pollen secondary metabolites

The SEM analysis identified interactions between *L. argenteus* interactors that were missing from the individual models (described in the sections above). The abundance of pollen beetles in flowers was positively associated with the abundance of thrips and pollen-colonizing bacteria (Fig. 3-5). However, incorporating these interactions in the SEM did not affect the conclusions from the individual models.

3.5 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 and 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 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.

3.5.1 Why are there defence compounds in pollen?

At face value, our results show mixed support for the pleiotropy hypothesis. Alkaloid concentrations in pollen were correlated 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 (Fig. 3-1A). 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 (Fig. 3-1B). 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 (Trunz et al., 2020). 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 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 non-adaptive mechanisms (Kessler and Halitschke 2009, Heiling et al. 2019, 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 selection for plants to protect their pollen against consumption (Palmer-Young et al. 2019, Heiling et al. 2019). Here, we found no support for a role of pollen secondary metabolites in protecting against two types of pollen thieves: thrips and pollen beetles (Fig. 3-2). Instead, thrips abundance was negatively associated with alkaloids in petals (Fig. 3-2B). 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 (Fig 3-1A). Pollen beetles from the genus *Eusphalerum*, which were not strongly deterred by either petals or pollen alkaloids in our study, are thought to consume exclusively pollen at the adult stage (Sayers et al. 2019). Although *Eusphalerum 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 defence 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, McFrederick and Rehan 2016, Kim et al. 2018, Manirajan et al. 2018). We found that the abundance of bacteria in pollen was negatively associated with pollen alkaloid concentration in the field (Fig. 3-3A). While this association could potentially originate from an indirect effect mediated by pollinators, which are important

dispersers of flower microbes (Russell et al. 2019, Zemenick et al. 2021, Vannette et al. 2022), our structural equation model (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 only recovers a small portion of the microbial diversity occurring on pollen (but see Ambika Manirajan. et al., 2016, for a study showing a good correspondence in the relative abundance of bacterial taxa recovered from cultivation-dependent and -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 defences of *Lupinus* pollen, while others, 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 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 (Vannette et al. 2012, Junker et al. 2014, Russell and Ashman 2019). 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 defences 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, Huang et al. 2012, Donati et al. 2018, Fetters et al. 2022), 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 that assess the direct impact of pollen microbes on pollination.

3.5.2 Are pollen defence compounds ecologically costly to plants?

Plant secondary metabolites involved in defence against antagonists are often deterrent to herbivores. These compounds could deter pollinators as well when they occur in pollen (Muth et al. 2016, Francis et al. 2019). This might be especially true for plants that depend exclusively on pollen to attract pollinators. The presence of defence 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 (Fig. 3-4b), suggesting that bees can detect alkaloids in pollen and are deterred by it. However, this impact on pollinator behaviour 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 (Fig. 3-4A). Pollen alkaloids therefore seem to affect pollinator behaviour, but not in a way that is likely to be costly to plant reproduction. It is also important to note that the negative association between pollen alkaloids and numbers 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 to our field observations, we found only weak evidence for a deterrent effect of pollen alkaloids in our laboratory behavioural assays (Fig. 3-4C, 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 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. Lastly, 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 resource to defence and to traits responsible for pollinator attraction (Strauss et al. 2002), although we could not test this possibility.

The absence of reproductive cost of pollen defensive compounds in terms of total numbers 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. This being 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 behaviour 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 and Nilsson 1999, Kessler et al. 2012). Notably, nectar secondary metabolites have been shown in a few instances to serve such a function (Kessler and Baldwin 2007, Kessler et al. 2012). Perhaps pollen secondary metabolites serve a similar function in

Lupinus by reducing geitonogamy. Future studies could directly test this hypothesis by tracking pollen dispersal from plants with various concentrations of pollen secondary metabolites, for example by using fluorescent dye or quantum dots (Adler and Irwin 2006, Minnaar and Anderson 2019).

3.5.3 Pollen defences 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 (Fig. 3-5). The best SEM demonstrated a direct impact of pollen alkaloids on pollinator behaviour and bacterial abundance, while pollen alkaloid concentration was correlated with petal alkaloid content, suggesting pleiotropy or spillover. In turn, petal alkaloids were negatively associated with the abundance of thrips, suggesting that thrips indirectly influence the concentration of alkaloids in pollen via the selective pressure that they might exert on petal alkaloids. This illustrates that pollen defensive compounds not only affect interactions with pollinators, but also with multiple non-pollinator interactors. Floral traits involved in multiple interactions with both mutualists and antagonists, such as pollen defences, 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 selection on correlated traits) could both directly and indirectly drive higher concentrations of pollen defence 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 defences, especially how the impacts of different drivers, both mutualistic and antagonistic, varies within and among species. Studies are needed that directly test the effect of pollen defences on plant fitness (for example by spiking pollen with secondary metabolites in the field), and that manipulate the abundance of multiple flower interactors.

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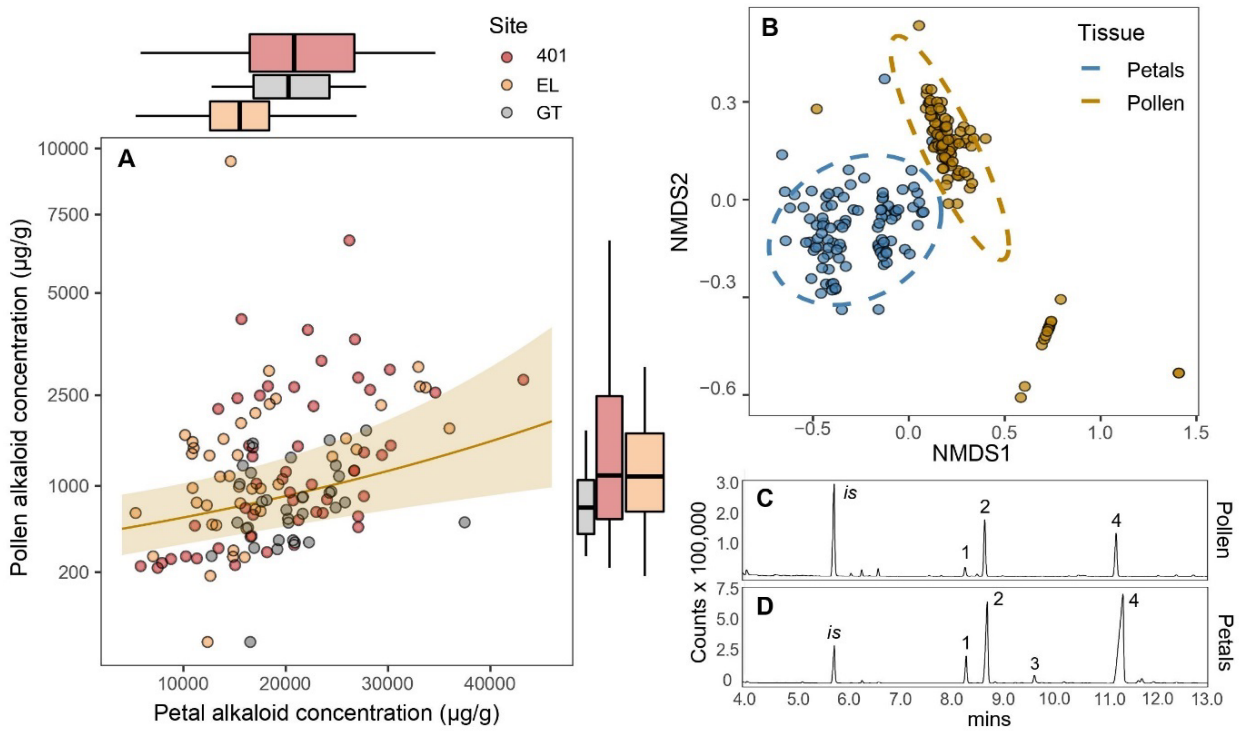

Figure 3-1 Concentration and composition of quinolizidine alkaloids in the pollen and petals of *Lupinus argenteus*. A) Relationship between levels of alkaloids in petals and pollen, showing values for the three study sites. Note that pollen alkaloid concentration is shown on a square-root scale. B) Nonmetric multidimensional scaling 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 Fig. 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, 2: α-Isolupanine, 3: 11,12-Dehydrolupanine, 4: thermopsine, *is*: internal standard (caffeine).

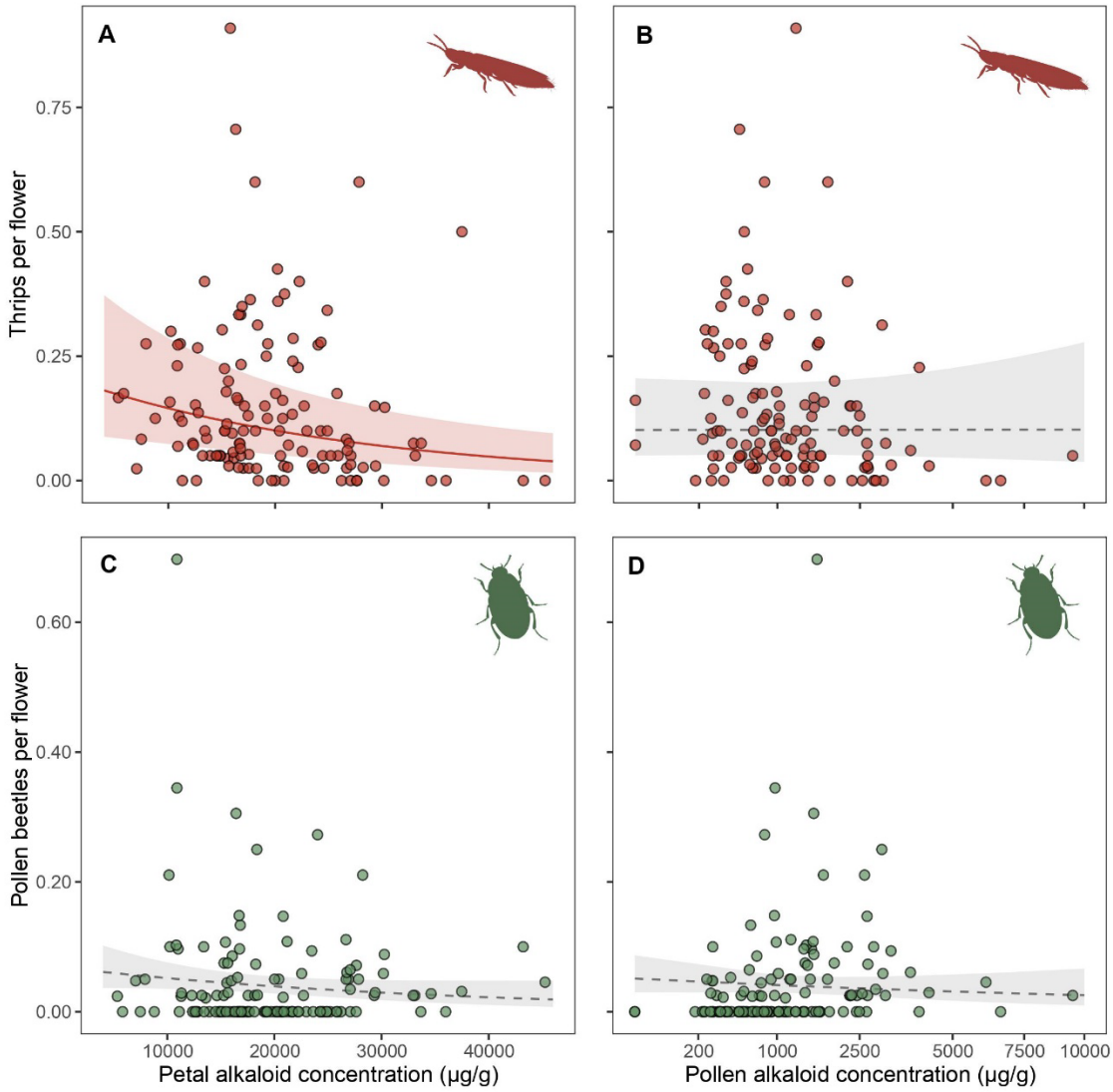

Figure 3-2 Relationships between *Lupinus argenteus* pollen and petal alkaloid concentrations and the abundance of two taxa of pollen thieves: A, B) thrips, and C, D) pollen beetles. Lines show predictions from the models, and coloured lines show significant associations.

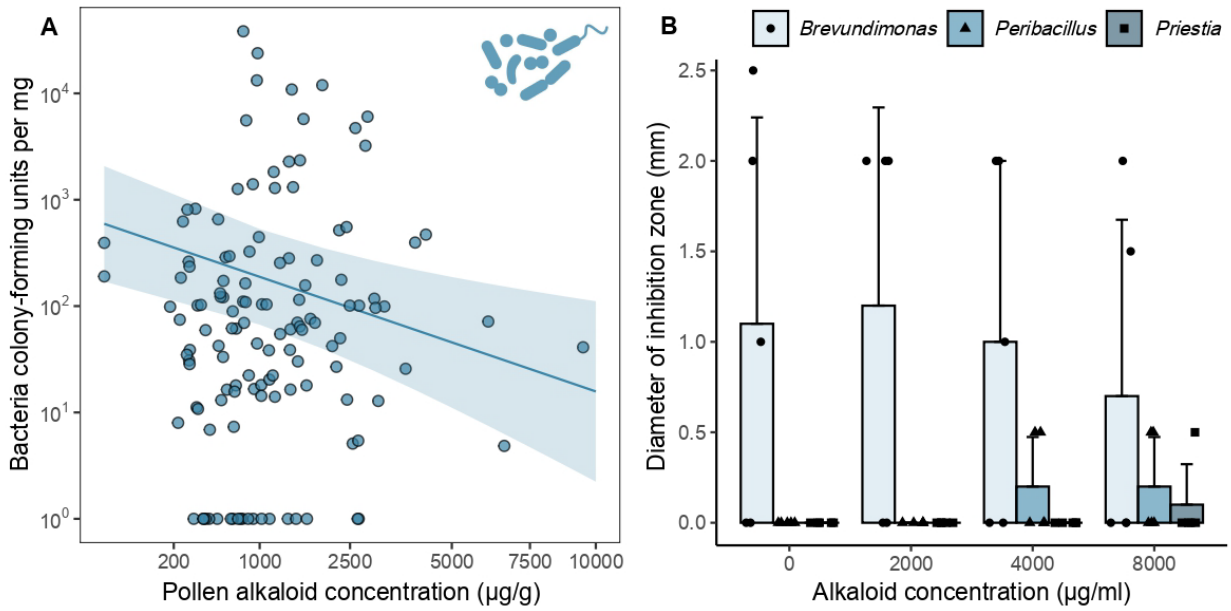

Figure 3-3 Antimicrobial effect of *Lupinus argenteus* pollen alkaloids. A) Relationship between *L. argenteus* pollen alkaloid concentration and the abundance of pollen-colonizing bacteria. B) Inhibitory effect of different concentrations of the pollen alkaloid thermopsine (mean + SD) on three species of bacteria isolated from *L. argenteus* pollen: *Brevundimonas* sp., *Priestia* sp., and *Peribacillus* sp.

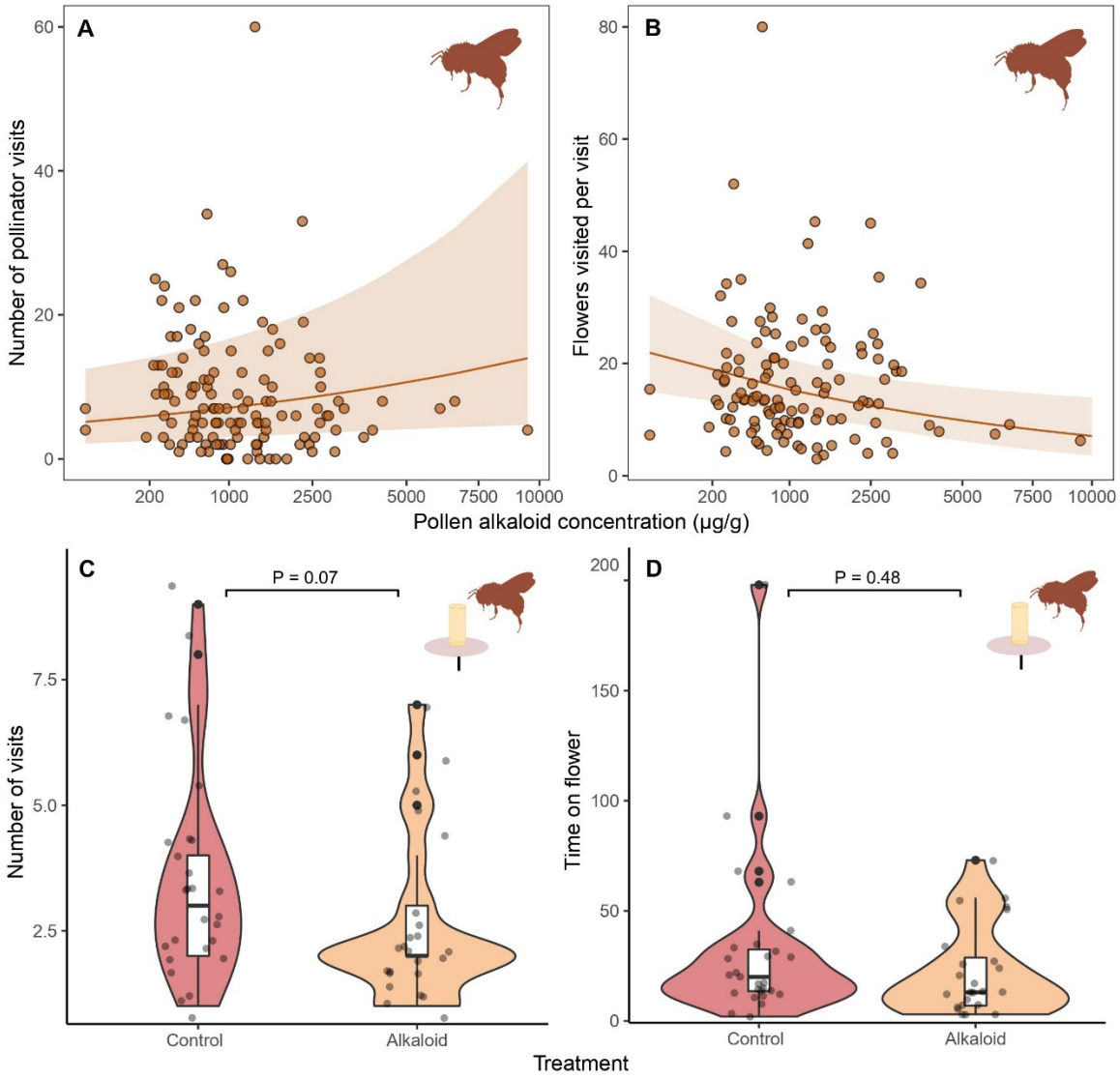

Figure 3-4 Relationships between *Lupinus argenteus* pollen alkaloid concentration and two components of pollinator visitation in the field: A) number of pollinator visits and B) average number of flowers visited per visit. Effect of the pollen alkaloid thermopsine (2 mg/g) on two components of *Bombus* visitation to artificial flowers in the laboratory: C) number of visits to artificial flowers and D) time spent on flowers.

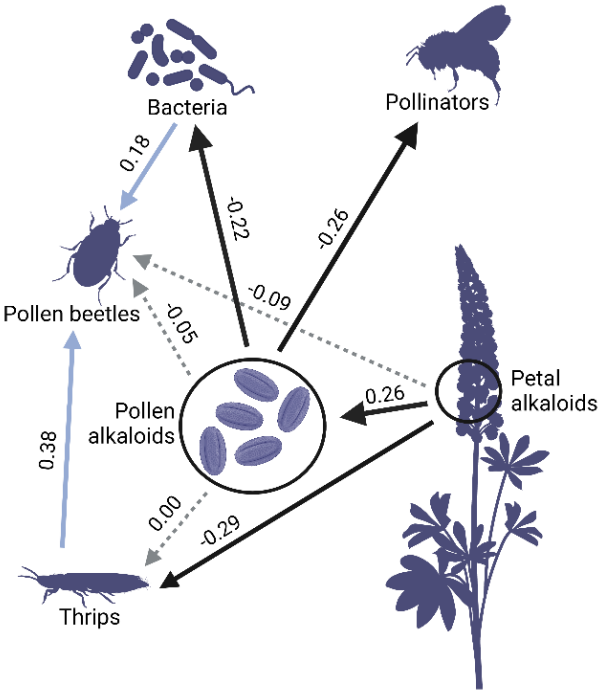

Figure 3-5 Path diagram showing the final structural equation model of the interactions between pollen and petal alkaloid concentration, and *Lupinus argenteus* interactors. Values of the standardized coefficients are given for each relationship and significant relationships after correcting for multiple comparisons are denoted by solid black lines. Pale blue lines represent missing paths from the original model that were incorporated in the final model.

3.7 Supporting information

3.7.1 Chemicals and reagents

Ammonium hydroxide, sodium sulfate, and chloroform were purchased from Fisher Scientific (Pittsburgh, PA), Baker (Phillipsburg, NJ), and Mallinckrodt Baker (Paris, KY), respectively; caffeine and sparteine from Sigma-Aldrich Chemical Co. (St. Louis, MO and Milwaukee, WI); lupanine from Biomedical Research Co. (Los Angeles, CA); and D-α-isolupanine perchlorate from Koch-Light Laboratories Ltd. (Colnbrook, Bucks, UK).

3.7.2 Alkaloid extraction

Petal and pollen samples were air-dried. Petal samples were first ground to pass through a 2 mm screen. Ground petal samples (25.0 mg) were transferred into 16 mL screw-top glass test tubes for extraction. Pollen samples were weighed into 16 mL screw-top glass test tubes for extraction. The samples were extracted by rotating the samples using a Roto-Shake Genie (Scientific Instruments, Inc; Bohemia, NY) with a mixture of 1 N HCl (4.0 mL) and $CHCl_3$ (4.0 mL) for 15 min (as reported in Lee et al. 2007). The samples were then centrifuged (5 min) and the aqueous layer removed. To each test tube containing sample and $CHCl_3$ an additional 2.0 mL of 1N HCl was added and followed by extraction by rotation (15 min), centrifugation, and removal of the aqueous layer. The aqueous portions were combined into clean, 16 mL screw-top glass test tubes and the samples basified to pH 9.0–9.5 by dropwise addition of NH_4OH with intermittent shaking of the test tube. The basified solution was then extracted twice with $CHCl_3$, first with 4.0 mL and then with 2.0 mL. The $CHCl_3$ was removed from each extraction, combined and filtered through anhydrous Na_2SO_4 into clean 7 mL screw-top glass test tubes. The $CHCl_3$ was then evaporated from each sample under N_2 at 60°C. Petal samples were reconstituted in MeOH (1

mL, containing 1.3 μg/mL caffeine internal standard). Pollen samples were reconstituted in the same MeOH internal standard solution with a volume proportional to the mass of the pollen. An aliquot of the reconstituted extract was then transferred to GC autosample vials for GC/FID or GC/MS analysis.

3.7.3 GC/FID analysis

Samples were analyzed by GC/FID using a Shimadzu GC-2010 gas chromatograph equipped with a Shimadzu AOC-20i autosampler, a J&W DB-5 column (30 m x 0.32 mm, 0.25 μm film thickness) and a flame ionization detector (FID). Samples (1.0 μL) were injected splitless at 250°C and used helium as the carrier gas at a constant flow rate of 2.0 mL/min. The column oven was temperature-programmed starting 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. Alkaloid concentrations were calculated from a five-point sparteine standard curve (25–500 μg/ml). Alkaloid concentrations are expressed as μg of sparteine per gram of plant tissue as standards were not available for all alkaloids.

3.7.4 GC/MS analysis

GC/MS analysis was performed as previously reported by Lee et al. (2007). In brief, two representative petal samples and two representative pollen samples from each population were analyzed by GC/MS using a Finnigan MAT GCQ equipped with a split/splitless injector and a DB-5MS (30 m x 0.25 mm; J&W Scientific) column. Samples (2.0 μL) were injected splitless at 250°C. The split vent flow rate was 50 mL/min was purged after 0.80 min. The column oven was temperature-programmed starting at 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. Electron impact ionization (EI) was at 70 eV with an Ion source temperature of 200°C.

3.7.5 Alkaloid identification

Alkaloid identification was performed as previously reported by Lee et al. (2007). In brief, five individual alkaloids were identified from authenticated (MS, NMR) samples of anagyrine and thermopsine from the alkaloid collection of the Poisonous Plants Research Laboratory (Logan, UT) and from commercially obtained standards (sparteine, lupanine, and D-α-isolupanine). Additional alkaloids were identified from correlation of measured retention times 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 mass spectra (Wink et al. 1995). In addition, alkaloid identification was further supported by correlation of measured relative retention times (RR_t) to lupanine and EI mass spectra to those reported in the literature (Kinghorn and Balandrin 1985).

Table 3-S1 Coordinates and elevations of the sites used for *Lupinus argenteus* sampling and for bumble bee nest collection.

Site	Experiment	Latitude (N)	Longitude (W)	Elevation (m)
401	*Lupinus* sampling	39°00′40″	107°01′49″	3376
EL		39°00′31″	107°02′29″	3189
GT		38°57′15″	106°59′21″	2872
IR	Bumble bee nest collection	38°52′34″	107°06′00″	3155
KP		38°51′10″	107°06′02″	3053

Table 3-S2 Concentration (μg/g) of alkaloid compounds in *Lupinus argenteus* petals and pollen at three sites in the Colorado Rocky Mountains. Concentration values correspond to the mean ± 1 standard deviation. − indicates the alkaloid was not detected; * indicates the alkaloid was detected in < 75% of samples.

Alkaloid	Petals			Pollen		
	401	EL	GT	401	EL	GT
α-Isosparteine	27 ± 37*	59 ± 60*	−	−	1 ± 7*	−
5,6-Dehydro-α-lupanine	1348 ± 584	1090 ± 552	−	168 ± 183	152 ± 145	−
α-Isolupanine	13720 ± 6237	7598 ± 3152	−	1164 ± 1148	867 ± 1001	−
11,12-Dehydrolupanine	694 ± 530	583 ± 277	−	−	−	−
Thermopsine	6688 ± 2490	7668 ± 4534	−	303 ± 262	444 ± 406	−
Sparteine	2 ± 12*	−	7 ± 18*	−	−	−
β-Isosparteine	−	−	17 ± 57*	−	−	−
Aphyllidine	−	−	606 ± 360	−	−	−
5,6-Dihydro-α-lupanine	−	−	422 ± 229	−	−	−
Lupanine	−	−	60 ± 52*	−	−	−
Argryolobine	−	−	3610 ± 2181	−	−	89 ± 95*
Dihydroxyaphylline	−	−	5614 ± 1994	−	−	103 ± 59
Unknown	−	−	673 ± 1011	−	−	34 ± 122*
Anagyrine	−	−	9800 ± 4032	−	−	568 ± 348

Figure 3-S1 *Bombus appositus* workers foraging on artificial flowers used to feed pollen (right and left) and nectar (middle) during training in a flight cage. Photo by Sébastien Rivest.

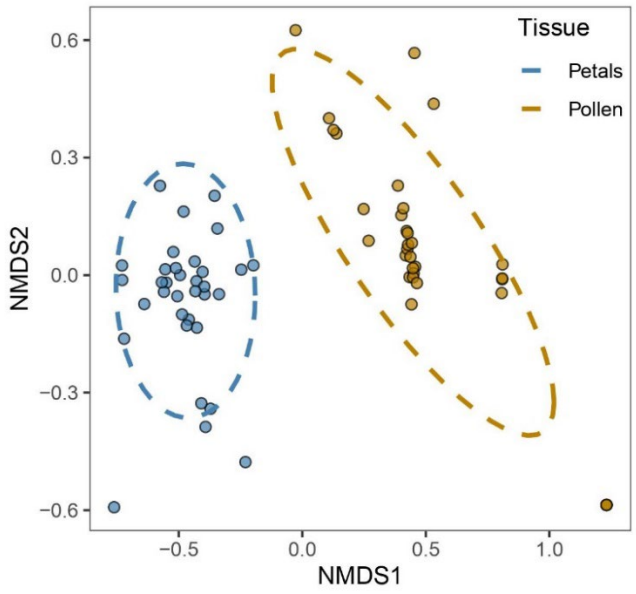

Figure 3-S2 Nonmetric multidimensional scaling ordination plot of the alkaloid profile in petals and pollen from the GT site (number of alkaloids = 9, stress = 0.08). Ellipses are drawn at a confidence level of 0.95.

Chapter 4: Pollen chemical and mechanical defences restrict host-plant use by bees

4.1 Abstract

Plants produce an array of chemical and mechanical defences that provide protection against many herbivores and pathogens. Putatively defensive compounds and structures can even occur in floral rewards: for example, the pollen of some plant taxa contains toxic compounds or possesses conspicuous spines. Yet little is known about whether pollen defences restrict host-plant use by bees. In other words, do bees, like other insect herbivores, tolerate the defences of their specific host-plants while being harmed by non-host defences? To answer this question, we compared the effects of a chemical defence from *Lupinus* (Fabaceae) pollen and a putative mechanical defence (pollen spines) from Asteraceae pollen on larval survival of nine bee species in the tribe Osmiini (Megachilidae) varying in their pollen host use. We found that both types of pollen defences reduce larval survival rate in some bee species. These detrimental effects were, however, mediated by host-plant use, with bees being more tolerant of the pollen defences of their hosts, relative to the defences of plant taxa exploited by other species. This pattern strongly suggests that bees are adapted to the pollen defences of their hosts, and that host-plant use by bees is constrained by their ability to tolerate such defences.

4.2 Introduction

Plants have developed an array of chemical and mechanical defences in response to selection imposed by herbivores and pathogens (Futuyma and Agrawal 2009, Moore et al. 2014, Richards et al. 2015). While these defences are often associated with vegetative tissues and mediate antagonistic interactions, nectar and pollen—the most common floral rewards—also frequently

contain toxic secondary metabolites (Stevenson et al. 2017, Palmer-Young et al. 2019, Rivest and Forrest 2020, Trunz et al. 2020), and many types of pollen exhibit conspicuous spines (Pope 1925, Tellería 2017, Konzmann et al. 2019). The concentration of secondary metabolites in pollen can even match that of vegetative tissues (Rivest and Forrest 2020). Therefore, not only herbivores in the classical sense (i.e., consumers of vegetative tissue), but also potentially pollinators, must confront plant defences. In insect herbivores, host-plant use is strongly restricted by plant defences, as herbivores often possess physiological adaptations allowing them to overcome some host defences, while they are harmed by non-host defences (Cornell and Hawkins 2003, Opitz and Müller 2009, Ali and Agrawal 2012). Indeed, plant defences are thought to be the main drivers of host-plant associations in herbivorous insects (Ehrlich and Raven 1964, Futuyma and Agrawal 2009). Given the frequent occurrence of seemingly defended floral rewards, it seems possible that host-plant associations of pollinators could be similarly constrained by their ability to tolerate plant defences.

Dietary specialization among the world's ~20 000 bee species is highly variable, and bee larvae differ greatly in their ability to develop to maturity on different pollen hosts, suggesting that as-yet unknown factors restrict pollen (but not nectar) use in these pollinators (Wcislo and Cane 1996, Praz et al. 2008, Sedivy et al. 2008, 2011, Eckhardt et al. 2014, Vanderplanck et al. 2018). In a few instances, pollen defence compounds have been found to harm or even kill bees that are not specialists on the plant taxa that produce these defences (Arnold et al. 2014, Trunz et al. 2020, Cane et al. 2020). Moreover, the patterns of host-plant use by bees, such as widespread specialization and phylogenetic conservatism (the tendency for related species to use similar hosts), show striking similarities to those of other herbivorous insects (Sipes and Tepedino 2005,

Sedivy et al. 2008, 2013, Müller and Kuhlmann 2008), indicating that host use could be influenced by similar plant characteristics (e.g., plant defences).

Bees' use of specific host-plant taxa for pollen can also influence their interactions with parasites (Spear et al. 2016, Palmer-Young and Thursfield 2017, Giacomini et al. 2018, Figueroa et al. 2023). Some bee parasites seem to be harmed by properties of some pollens, but in most cases, we do not know what the harmful properties are; they may or may not be the same ones that influence bee host-plant use. In herbivorous insects, plant defences are sometimes co-opted for defence against enemies (Opitz and Müller 2009, Erb and Robert 2016); this could also be true in bees (Spear et al. 2016).

Here we ask whether host-plant use by bees is restricted by plant defences—that is, do bees parallel other insect herbivores in being tolerant of the defences of their hosts and intolerant of non-host defences? To test this, we compare the ability of multiple bee species to tolerate pollen defences from their host-plants relative to those of co-occurring non-host plants exploited by other, closely related bee species. Specifically, we test the effect of a putative mechanical defence from Asteraceae pollen and a chemical defence from *Lupinus* (Fabaceae) pollen on larval development and survival in nine bee species of the tribe Osmiini, including generalists, specialists on Asteraceae pollen, and a specialist on Fabaceae pollen. A better tolerance of host vs. non-host defences is typically observed in insect herbivores, a pattern underlying the important role of plant defences in restricting host-plant use among herbivorous insects (Cornell and Hawkins 2003, Opitz and Müller 2009). From this pattern we can deduce that 1) insect herbivores are typically adapted to the defences of their hosts, and 2) there is a fitness cost to exploiting novel hosts. Finding a similar pattern in bees would suggest that host-use is similarly

restricted by plant defences. We also assess the effects of these putative defences on a pollen-feeding wasp that is the primary brood parasite of several Osmiini species, as pollen defences could protect bees adapted to tolerate such defences against parasites.

4.3 Methodology

4.3.1 Study species

The tribe Osmiini (Hymenoptera: Megachilidae) includes many pollen specialists and generalists (Rust 1974, Haider et al. 2014). As in most bees, dietary specialization in Osmiini occurs only for pollen, not for nectar, and is typically at the level of plant genera, tribes, or families (not individual plant species). Many species of Osmiini nest in abandoned tunnels made by other insects in dead or damaged trees. Osmiini nests consist of a series of individual brood cells, each containing a single egg laid on a pollen-and-nectar provision. The brood cells are produced sequentially along the length of the nest tunnels and are separated by walls made of mud and/or plant material (Cane et al. 2007). In osmiine bees, mothers provide all the larval food before the larvae hatch; thus, mothers cannot adjust the content of pollen provisions in response to larval health.

We studied nine Osmiini species, all of which readily nest in artificial nesting cavities ("trap-nests"; see below), facilitating nest collection and experimentation. This included five generalist species (*Hoplitis fulgida* (Cresson)*, Osmia lignaria* Say, *O. pusilla* Cresson*, O. tersula* Cockerell, *O. tristella* Cockerell), three Asteraceae-specialists (*O. coloradensis* Cresson, *O. montana* Cresson, *O. subaustralis* Cockerell), and one Fabaceae-specialist (*O. iridis* Cockerell & Titus). The brood cells of four of these species (*O. lignaria*, *O. tersula*, *O. tristella*, and *O. iridis*) were frequently parasitized by the brood parasite *Sapyga* sp. (Hymenoptera: Sapygidae;

misidentified as *S. pumila* in previous publications), larvae of which kill the host egg before consuming its food provision (Spear et al. 2016). This brood parasite was therefore also included in our experiments. Bee diets were characterized by examining the pollen content of hundreds of nests over 8 years (Fig. 4-S1; see Spear et al. 2016 for details) and consulting relevant literature (Rust 1974, Barthell et al. 1997, Wilson et al. 2010, Haider et al. 2014, Roof et al. 2018). We considered a species to be a pollen specialist (i.e., oligolectic) when more than 95% of the pollen provision samples examined were dominated by a single plant family.

4.3.2 Nest collection

We collected Osmiini nests using artificial nesting structures that consist of wooden blocks containing holes of three different diameters (to attract different species of bees), affixed to dead or dying trees to mimic the natural cavities in which Osmiini nest (see Forrest and Chisholm 2017 for details). We lined the holes of the nesting structures with blind-ended paper straws that could be removed to collect bee nests without damaging them. We installed between 6 and 12 artificial nesting structures at each of 12 sites near the Rocky Mountain Biological Laboratory in Colorado, USA (see Table 4-S1 for site locations).

4.3.3 Pollen defence manipulations

To test the effect of pollen defences on bee larval survival and development, we selected two types of pollen defences from plants that are 1) abundant at our study site, and 2) exploited by some of the investigated Osmiini species but avoided by others. Specifically, we tested the effect of Asteraceae pollen spines and *Lupinus* pollen alkaloids. Asteraceae pollen, like that of a few other plant families, is characterized by conspicuous spines on its surface (Fig. 4-1E). The exact role of pollen spines in pollination remains unclear, but one study provided evidence that they

can deter generalist pollinators (Lunau et al. 2015). Among our study species, Asteraceae pollen is exploited by Asteraceae specialists, but is rarely observed in the provisions of generalists and Fabaceae specialists (Fig. 4-S1; see also Praz et al. 2008, Müller and Kuhlmann 2008).

The plant genus *Lupinus* (Fabaceae) contains toxic quinolizidine alkaloids in most of its tissues, including its pollen (Detzel and Wink 1993, Arnold et al. 2014, Heiling et al. 2019). Based on pollen sampling from four populations by Heiling et al. (2019), the population average concentration of quinolizidine alkaloids in *L. argenteus* pollen ranges from 1.51 to 4.03 mg/g (overall population average = 2.81 mg/g). The main quinolizidine alkaloids in *L. argenteus* pollen near our study site are thermopsine, anagyrine, and α-isolupanine (Heiling et al. 2019; Fig. 4-1D). *Lupinus* pollen is common in the provisions of our investigated Osmiini generalists, ranging from being a minor contributor to being the principal pollen, but it was not observed in the provisions of the Asteraceae and Fabaceae specialists (Figs. 4-S1-S2). The Fabaceae specialist (*O. iridis*) appears to exclusively use pollen from the tribe Fabeae (*Lathyrus* and *Vicia* spp. in our study area), despite the similarity of the latter in flower size and morphology to *Lupinus*.

We separated the experiment into two sub-experiments: one for the Asteraceae pollen spines and one for the *Lupinus* alkaloids. All provisions within a nest (i.e., contained within the same paper straw) were assigned to the same sub-experiment. In both sub-experiments, provisions were assigned alternately to the control and defence-addition treatments. Prior to weighing and manipulating the provisions, eggs (or young larvae; see below) were separated from their provisions using spatulas and were stored in empty Petri dishes with moist cotton balls to maintain high humidity. After manipulation of provisions, the eggs were placed back on their

own provisions, usually within 1.5 hours of being separated from the provisions. Eggs were used when possible, but young larvae (within 10 days of the estimated date on which the egg was laid; in our study area, eggs hatch approximately 1 week after being laid) were sometimes used as well. Brood cells parasitized by *Sapyga* sp., in which the *Sapyga* larva had replaced the host, were also manipulated following the same methodology as for Osmiini.

To manipulate the presence of Asteraceae pollen spines in Osmiini provisions, we added to the provisions empty pollen "shells" of either Asteraceae pollen or, for our control, a mix of non-spinous pollen grains, both subjected to the same procedure. This manipulation allowed us to remove potential confounding effects of pollen nutrients and chemical defences on bee larval development. Honeybee-collected pollen was used in both treatments (Asteraceae pollen was purchased from Changge Huading Wax Industry Co, Henan Province, China; non-spinous pollen was purchased from Alovitox, California, United-States). We visually confirmed that the pollen used for the Asteraceae-spines treatment contained more than 95% Asteraceae pollen, while the pollen used for the non-spinous treatment contained less than 5% spinous pollen grains (using microscope slides stained with basic fuchsine). We first removed sugar from the honeybee-collected pollen by washing it in deionized water (following McAulay and Forrest 2019). We then washed the pollen grains in warm ethanol twice to remove the lipidic external layer of the pollen grains (i.e., pollenkitt). We finally removed the inner content of the pollen grains using hot acid reflux with phosphoric acid for 5 hours followed by a series of 17 wash steps with solvents (ethanol and acetone), hydrochloric acid, and water (following Fan et al. 2018). We added empty pollen shells (exine) to the provisions at a concentration of 5% by mass, corresponding to ~40% by volume (empty exine is considerably lighter than unmanipulated pollen grains). We verified that the empty pollen shells of both treatments occupied

approximately the same volume by making microscope slides stained with basic fuchsine of the pollen content of the manipulated provisions (using the same method as for the determination of the pollen content of the provisions). The percent volume of empty pollen shells in the provisions was measured using the image processing software ImageJ (Schneider et al. 2012). Because Osmiini pollen provisions are made of a mix of pollen and nectar (in proportions that vary among species, but with more pollen than nectar; Williams 2003, Radmacher and Strohm 2010), we added sterile sugar water (30% sugar, using table sugar) to the provisions to compensate for the volume of empty pollen shells added to the provisions. Sterile sugar water was added in a concentration of 0.15 µl/mg of the initial provision (before adding the empty shells). This allowed us to obtain a consistency comparable of that of natural provisions. The provisions were mixed with the added pollen shells and sugar water to obtain a homogeneous texture.

To manipulate the presence of *Lupinus* chemical defence we spiked the provisions with a mix of thermopsine and α-isolupanine in a 5:1 ratio and a concentration of 2 mg/g of provision (anagyrine, another of the most commons alkaloids in *L. argenteus* pollen, is a stereoisomer of thermopsine). This concentration was within the range of quinolizidine alkaloid content in the pollen of *L. argenteus* (see above). Because Osmiini provisions are mainly composed of pollen (Williams 2003, Radmacher and Strohm 2010), and because *Lupinus* pollen was sometimes the main pollen component in unmanipulated provisions of the generalist species, it seems likely that generalist Osmiini are frequently exposed to the concentration of *Lupinus* alkaloids used in this study. The alkaloids were dissolved in ethanol and the solution mixed with the provisions to allow the alkaloids to be homogeneously distributed. We used a higher proportion of thermopsine than α-isolupanine (rather than mimicking the exact ratio observed in pollen)

because the latter had a lower solubility in ethanol. We added ethanol (without alkaloids) to the control provisions. In both treatments, ethanol was allowed to evaporate from the provisions for one hour before eggs were reintroduced.

Following manipulations, the eggs with their provisions were stored in individual wells (~2 cm × 1 cm) in wood blocks. A glass coverslip was taped on the top of each well to allow observation of larval survival and development. The wood blocks containing the eggs or larvae were kept in a growth chamber on a 10–26 °C ramping diurnal cycle and assessed every other day to monitor the bees' survival and developmental stage. Survival was assessed until larvae started spinning their cocoons, at which point they had reached the final (fifth) larval instar and had usually stopped feeding.

4.3.4 Phylogenetic reconstruction

To be able to account for phylogenetic relatedness in our statistical analyses, we constructed a phylogeny for our investigated Osmiini species using the concatenated and aligned matrix from a previously published *Osmia* phylogeny (Rightmyer et al. 2013), which was constructed using DNA sequence data from three nuclear genes (elongation factor 1-α, LW-rhodopsin, and CAD) and the mitochondrial COI gene. The phylogeny of Rightmyer et al. (2013) included most of our *Osmia* species, but it did not include *Hoplitis fulgida* or *O. tersula*. However, it did include close relatives of each of these species, so we were able to use the sequence data from those close relatives as proxies: For *H. fulgida*, which was the only *Hoplitis* species in our study, we used sequence data from the closely related *H. albifrons* (*H. albifrons* and *H. fulgida* belong to the same subgenus). For *O. tersula*, we used data from *O. inermis*, which was identified as the nearest neighbour of *O. tersula* by Sheffield et al. (2009) (both species belong to the same

subgenus). (We could not use the Sheffield et al. (2009) phylogeny for our analyses because it did not include most of our study species.) Our results were robust to the removal of *O. tersula* or *H. fulgida*, suggesting that potential errors in branch lengths for these species did not affect our results. We inferred a phylogeny for our nine Osmiini species from the Rightmyer et al. dataset under the Maximum Likelihood method on RAxML v.8 (Stamatakis 2014) and calculated bootstrap proportions via 1000 replicates. The estimated phylogeny was congruent with the tree of Rightmyer et al. (2013) and had high branch support (>95%). We did not pursue a Bayesian estimation of the phylogeny since the same dataset was found by Rightmyer et al. (2013) to provide robust estimations under both ML and Bayesian methods. We determined the phylogenetic correlation among Osmiini species using the vcv.phylo function from the R package ape (Paradis et al. 2004).

4.3.5 Statistical analysis

We conducted all analyses in R version 4.1.1. (R Core Team 2020). We used Bayesian Cox-proportional hazard models to test the effect of pollen chemical and mechanical defences on bee larval mortality rate. Using Bayesian models allowed us to test the effect of the pollen defences on each bee species simultaneously without introducing problems of multiple testing, while also controlling for phylogenetic relatedness (see below). To compare the effect of the pollen defences among bee species, we first tested the effect of the two pollen defences (in separate models) on all bee species, irrespective of their pollen host. Pollen defence treatment was included as a fixed effect. As random effects, we included the species identities with their phylogenetic co-variance with random intercepts and random slopes (the random slopes allow the effect of the pollen defence treatment to vary between species), and the nest's source location (nest, nested within nesting block, nested within site) with random intercepts. To test if pollen

host-use mediates the ability of bees to tolerate pollen defences, we used another set of models that compared the effect of the pollen defences between pollen-host-use types (Asteraceae specialists, Fabaceae specialists, and generalists). We included the pollen defence treatment (control or pollen defence), the pollen-host use of the bee, and an interaction term between those two variables, as fixed effects. The interaction between treatment and pollen-host use allowed us to test if bees' abilities to tolerate pollen defences depend on their pollen-host use. We included the species identities with their phylogenetic co-variance, and the nest location (as above) as random effects.

The models were run using Hamiltonian Monte Carlo (HMC) sampling, with four chains of 10,000 iterations of which the first 2500 were discarded as burn-in. We used normal priors (mean = 0, s.d. = 5) for the fixed effects and Student-t priors (location = 0, degrees of freedom = 3, scale = 2.5) for the variance of the random intercepts and slopes. These values were chosen to be weakly informative (i.e., slightly regularizing the model without providing information about the expected outcome; see McElreath 2020). The thinning intervals were set to 8 to reduce autocorrelation in the Markov chains. Convergence of chains for all parameters was verified both visually with trace plots and with the Gelman–Rubin convergence statistic (Gelman and Rubin 1992). The models were run using the brm function from the brms package in R (Bürkner 2017).

4.4 Results and discussion

4.4.1 Pollen defences increase mortality in Osmiini

We first tested whether naturally occurring concentrations of pollen chemical defence from *Lupinus* and mechanical defence from Asteraceae are detrimental to larval survival in Osmiini. We found that both types of defences are harmful to some Osmiini species, with each defence

affecting a different subset of the investigated species. Specifically, *Lupinus* alkaloids increased the mortality rate of two Asteraceae-specialist Osmiini but had no noticeable impact on the mortality of generalists and Fabaceae specialists (Fig 4-2A). Conversely, Asteraceae spines were detrimental to the survival rate of Fabaceae specialists and some generalists, while other generalists and the Asteraceae specialists exhibited little to no impact from this putative defence (Fig 4-2B). In species harmed by pollen defences, the hazard ratio—the ratio in mortality rate between the treatment and control larvae—typically ranged from 4 to more than 10 (Fig. 4-2). These multiple-fold increases in mortality rate show that pollen defences can have substantial impacts on the fitness of Osmiini bees. Moreover, similar mortality rates are common in solitary bee larvae reared on non-host pollen, providing support for the hypothesis that pollen defences contribute to the frequently observed unsuitability of non-host pollen for bee larval development (Williams 2003, Praz et al. 2008, Sedivy et al. 2011, Vanderplanck et al. 2018, McAulay et al. 2020).

4.4.2 Osmiini better tolerate the defences of their hosts relative to non-host defences

Given that pollen defences can affect fitness in Osmiini, we then compared the ability of these bees to tolerate host relative to non-host defences. We found that the ability of bee species to tolerate different pollen defences is mediated by their host-plant use, with bees being more tolerant of the pollen defences of their hosts relative to defences from non-host plants exploited by other Osmiini species. Specifically, Asteraceae pollen spines were more harmful to Osmiini species that do not, or rarely, exploit Asteraceae pollen (Fabaceae specialists and generalists) relative to Asteraceae specialists (Bayesian credible interval of the differences in hazard ratio between host-use types: 95% CI = 1.21 to 2.95 for Fabaceae vs. Asteraceae specialists, and 0.66 to 2.28 for generalists vs. Asteraceae specialists) (Fig. 4-3D-F). Similarly, generalist Osmiini,

which often exploit *Lupinus* as a pollen host, better tolerated *Lupinus* pollen alkaloids than did Asteraceae specialists (CI = 0.77 to 3.43), although the Fabaceae specialists (which also do not use *Lupinus* pollen) were no more affected than generalists by this defence (CI = −0.84 to 2.09) (Fig. 4-3A-C).

Overall, the fact that defences from pollen hosts readily exploited by some bee species are harmful to other species suggests that, like other insect herbivores, bees are physiologically or morphologically adapted to the defences of their hosts (Sedivy et al. 2011, Gompert et al. 2015, Rivest and Forrest 2020). In turn, by showing that these pollinators often lack tolerance of non-host pollen defences, our results provide strong evidence that such defences constitute a constraint in bee pollen-host use. As in other insect herbivores, the exploitation of plant hosts in bees might often require overcoming certain detrimental effects of pollen chemistry and morphology, perhaps by evolving physiological adaptions to pollen properties or via behavioural mechanisms that reduce exposure to them (e.g., pollen mixing; Eckhardt et al. 2014).

Notwithstanding these general patterns, some Osmiini species were highly tolerant of non-host pollen defences. For example, while the two closely related Asteraceae specialists, *O. montana* and *O. subaustralis*, were detrimentally affected by *Lupinus* alkaloids, the more phylogenetically distant *O. coloradensis* tolerated these compounds (Fig. 4-2A). Similarly, the Fabaceae specialist *O. iridis* was not affected by alkaloids from *Lupinus*, a genus closely related to the floral hosts of *O. iridis*. The lack of effect of lupine alkaloids on *O. iridis* suggests that these bees avoid lupine flowers for other reasons, such as other chemical constituents of the pollen or the fact that lupine flowers do not produce nectar. The capacity of some Osminii species to tolerate non-host defences is not unexpected considering that this capacity frequently occurs in other insect

herbivores as well (Koenig et al. 2015, Larose et al. 2019). In both, the ability to use non-host plants is thought to occur when adaptations to ancestral hosts have not yet been lost despite transitions to new hosts having occurred. In bees, evidence for this mechanism of non-host tolerance comes from the fact that diet expansion often occurs through the adoption of pollen-hosts exploited by related species, suggesting that phylogenetically conserved adaptations are frequent (Sedivy et al. 2008).

Irrespective of treatment, larval mortality was higher in the Asteraceae-spines subexperiment than in the *Lupinus*-alkaloids experiment (Fig. 4-2). This difference likely occurred because in order to isolate the mechanical properties of pollen grains in the Asteraceae spines experiment, empty pollen "shells", rather than nutrient-filled pollen grains, were added to the provisions (at ~40% v/v), which reduces the nutrient concentration in larval provisions. This lower nutrient concentration is however unlikely to be responsible for the difference we observed between treatments, since the proportion of empty exine was similar between spiny and non-spiny (control) pollen treatments (5% m/m in both treatments, corresponding to v/v of 39.9 ± 5.0% for non-spiny and 36.4 ± 6.1% for spiny exine; see Methodology). In this experiment, Asteraceae-specialist Osmiini represented a good control to assess whether we effectively isolated the relevant mechanical properties of pollen grains because these species are expected to tolerate pollen spines relatively well. We found little to no detrimental impact of pollen spines relative to non-spinous pollen shells in these latter species (CI = −0.03 to 1.00, Fig. 4-2B, Fig. 4-3D-F), reinforcing our conclusion that pollen mechanical properties are responsible for the strong detrimental effect of spinous pollen exine on generalists and Fabaceae specialists.

4.4.3 Pollen defences protect specialist bees against a cleptoparasite

In addition to Osmiini, we investigated the impact of pollen defences on a common cleptoparasite of *Osmia* spp., *Sapyga* sp. In our study area, this *Sapyga* species frequently parasitizes Fabaceae specialists and generalists but does not occur on the provisions of Asteraceae specialists (Spear et al. 2016). Moreover, a previous egg-transfer experiment has shown that the mortality rate of *Sapyga* wasps is higher on Asteraceae provisions than on the provisions of Fabaceae specialists and generalists (Spear et al. 2016). Our results show that Asteraceae pollen spines increase the mortality rate of *Sapyga* wasps, suggesting that this pollen defence is responsible for the protective function of Asteraceae pollen against cleptoparasites (Fig. 4-2B). In contrast, we did not detect an effect of alkaloids from *Lupinus*, a pollen type frequently present in the provisions of *Sapyga*'s hosts, on *Sapyga* mortality (Fig. 4-2A). This finding provides evidence that specialization on putatively defended pollen hosts can confer fitness advantages in bees via its protective effect against antagonists. Wynns et al. (2012) found evidence for a similar protective function of the toxic protoanemonine from *Ranunculus* (Ranunculaceae) pollen against parasitic fungi in bees specialized on this pollen. Hence, parasites and pathogens could impose selection favouring specialization on defended pollen in bees, a pattern akin to the co-opting of defences by specialized herbivores (Opitz and Müller 2009, Erb and Robert 2016).

4.4.4 The role of pollen defences in the ecology and evolution of bees

Despite bees and other pollen consumers seemingly facing an array of defences, little is known about how these putatively antagonistic traits affect the ecology and evolution of pollinators. Here we demonstrate a striking parallel between bees and phytophagous insects: like phytophagous insects, bees tolerate the defences of a restricted subset of potential hosts while often being harmed by the defences of non-host plants. Moreover, in bees and in other

herbivorous insects, specialization on defended hosts can confer protection against antagonists. These findings, in combination with previous studies showing lethal or sublethal effects of pollen secondary metabolites on bees (Arnold et al. 2014, Trunz et al. 2020, Cane et al. 2020), suggest that pollen defences might be an important driver of pollen-host use in bees.

Multiple lines of evidence indicate that bees are highly constrained in their pollen diet. First, the fact that bees often specialize on specific hosts for pollen but rarely for nectar suggests that pollen requires more taxon-specific adaptations to acquire and metabolize (Wcislo and Cane 1996), a pattern that coincides with the typically higher concentration of secondary metabolites in pollen than nectar (Palmer-Young et al. 2019, Rivest and Forrest 2020). Perhaps more revealing is the remarkable similarity between the evolutionary patterns of host-use in bees and other insect herbivores, the latter being mostly attributed to plant defences (Ehrlich and Raven 1964, Radtkey and Singer 1995, Sipes and Tepedino 2005, Sedivy et al. 2008, 2013, Janz and Nylin 2008, Müller and Kuhlmann 2008, Futuyma and Agrawal 2009). This similarity is not only superficially suggestive; the evolutionary patterns in question—phylogenetic conservatism, widespread specialization, and the integration of new hosts exploited by related species—usually manifest when host-use is constrained by host characteristics of some kind (Radtkey and Singer 1995, Sedivy et al. 2008, 2013). For example, phylogenetically conserved host associations should occur when host use is not labile, leading to restricted ability to integrate new hosts or switch hosts over evolutionary timescales (Radtkey and Singer 1995, Janz and Nylin 2008, Rivest and Forrest 2020). Pollen defences should at least partially contribute to these patterns since they appear to restrict bees' ability to exploit new pollen hosts, although other factors such as pollen nutrients and bee neurological constraints may also play a role (Sedivy et al. 2008, Vaudo et al. 2016, 2020). In other words, the often-drastic increase in mortality associated with

feeding on non-host pollen that we observed across Osmiini species should limit the ability of bees to adopt novel hosts whose defences they do not tolerate. Our results therefore corroborate the hypothesis (Sedivy et al. 2008, 2011) that the striking similarity between the evolutionary patterns of host use in bees and other herbivorous insects is due to their diet being restricted by the same host characteristics: plant defences—both chemical and morphological. As we demonstrate in this study, pollen defences are not just a botanical curiosity, but rather likely play an integral role in the ecology and evolution of plants' main pollinators, bees. Considering pollination through this antagonistic perspective—by investigating the causes and consequences of pollen defences—promises to improve our understanding of the intricate interactions between plants and their pollinators.

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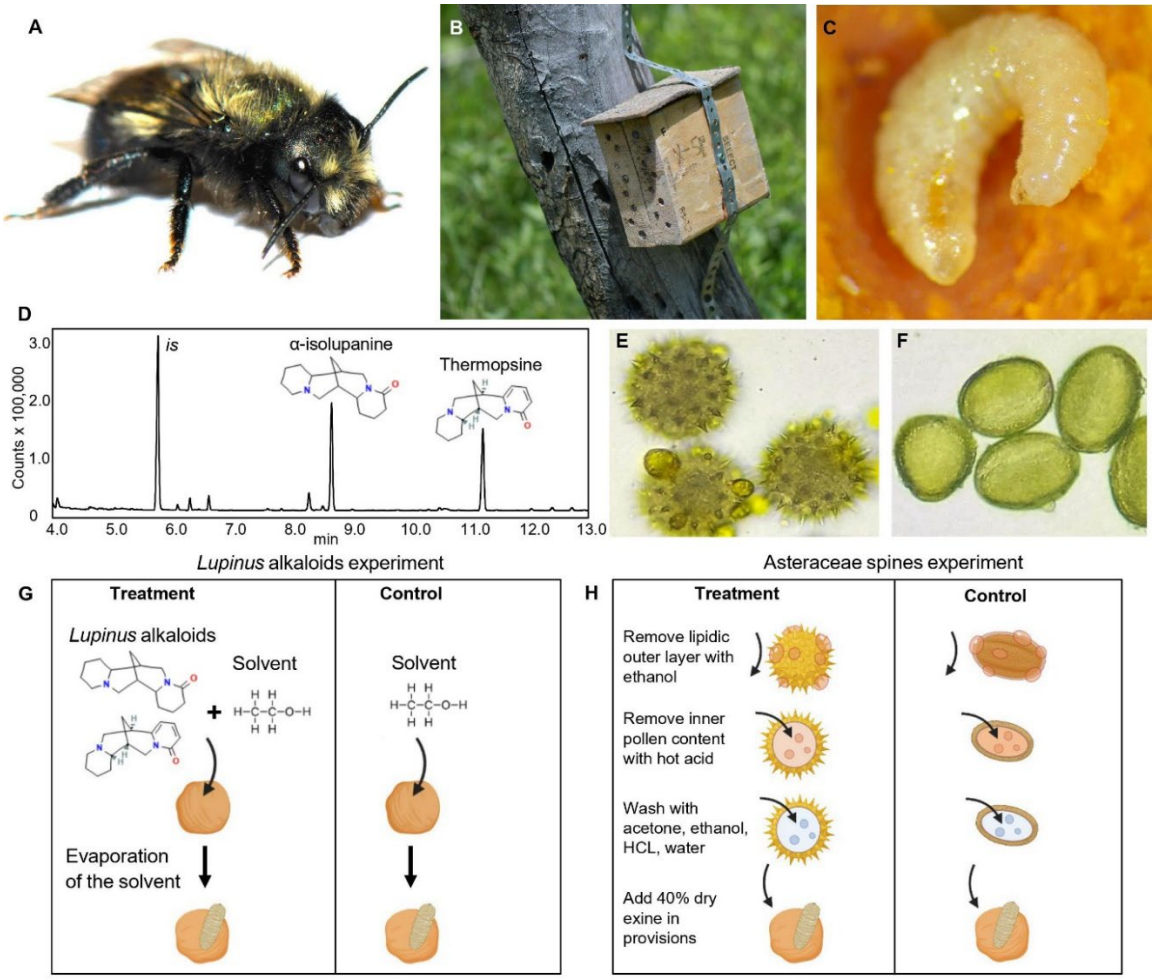

Figure 4-1 Types of pollen defences and experimental design for the larval provision manipulation experiment. A) *Osmia lignaria*, a generalist species of Osmiini. B) Artificial nesting structure containing holes lined with paper straws used to collect Osmiini nests. C) Larva of *O. montana* on its provision. Larval development and survival were recorded at least every other day. D) GC-FID chromatogram showing the pollen alkaloid profile of a *Lupinus argenteus* individual (a common host of generalist Osmiini) from our study site showing peaks for α-isolupanine and thermopsine (*is* = internal caffeine standard), two of the most common quinolizidine alkaloids in *L. argenteus* pollen. α-isolupanine and thermopsine were used to manipulate the presence of *Lupinus* pollen alkaloids in Osmiini provisions. E) Asteraceae pollen with visible spines. F) Non-spiny pollen used as a control in the Asteraceae pollen spines experiment. G) Methodology for manipulating the provisions in the *Lupinus* alkaloid experiment. H) Methodology for manipulating the provisions in the Asteraceae pollen spines experiment. Photos and illustrations by Sébastien Rivest.

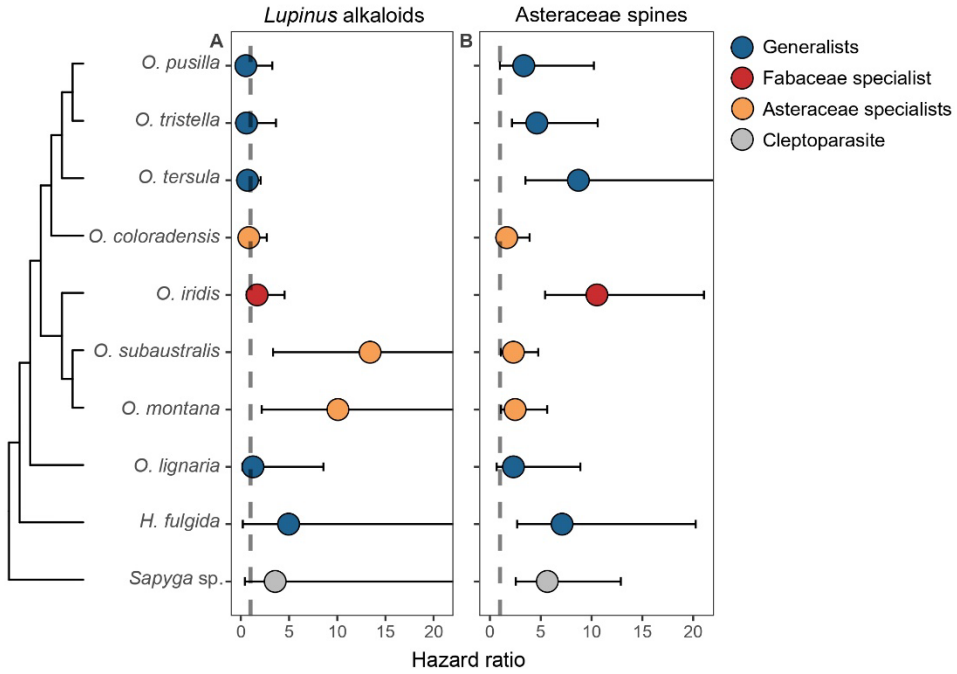

Figure 4-2 Effect of *Lupinus* chemical defence (A) and Asteraceae mechanical defence (B) on the mortality rate of nine Osmiini species as well as *Sapyga* sp., a cleptoparasite of *Osmia*. The effect of a pollen defence is represented as the hazard ratio: the ratio in mortality rate between larvae in the pollen defence treatment and those in the control, with larger values representing higher mortality in the treatment relative to the control larvae. The mean predicted values and the 95% Bayesian credible interval are presented (the interval extends beyond the limits of the figure for *O. subaustralis*, *O. montana*, *H. fulgida*, and *Sapyga* sp. in A) and for *O. tersula* in B)).

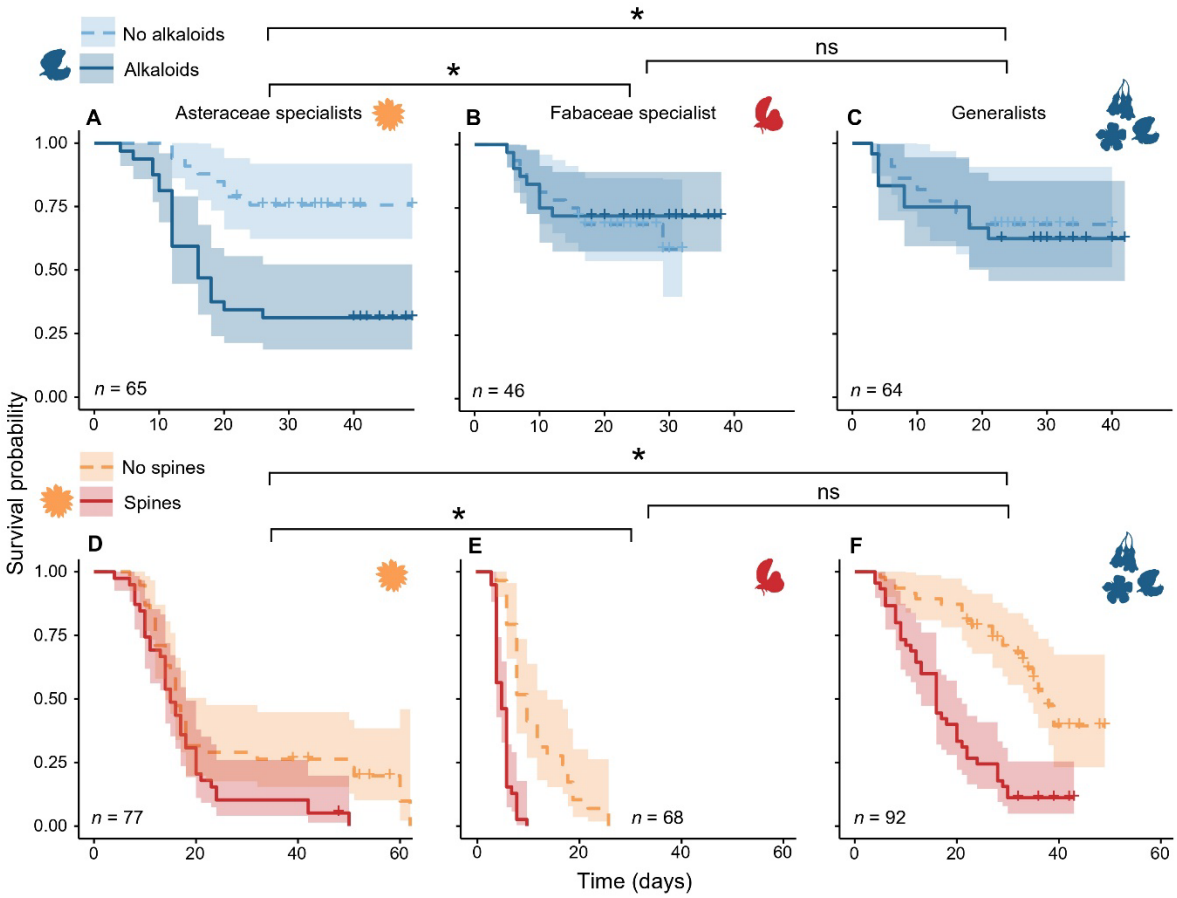

Figure 4-3 Survival curves of Osmiini bees exposed or not to *Lupinus* chemical defence (A-C) or Asteraceae mechanical defence (D-F) as a function of their host use types: Asteraceae specialists (A, D), Fabaceae specialists (B, E), and generalists (C, F). Significant differences (95% Bayesian credible interval does not overlap zero) in tolerance of a given defence between host-use types are represented by asterisks.

Table 4-S1 Collection sites for osmiine bees used in the experiment.

Site name	Latitude (N)	Longitude (W)	Elevation (m)
Brush Creek	38°51′45″	106°55′02″	2,750
Rosy Point	38°55′54″	106°58′01″	2,880
Gothic Townsite	38°57′31″	106°59′11″	2,908
South Gothic	38°57′14″	106°58′59″	2,938
Kebler Clearing	38°51′31″	107°03′38″	2,956
Judd Falls	38°57′42″	106°59′05″	2,988
401 Trail	38°58′15″	107°06′04″	3,020
Snodgrass	38°55′21″	106°58′13″	3,025
Kebler Pass	38°51′09″	107°06′04″	3,040
Virginia Basin	38°58′36″	106°58′37″	3,427
Baldy	38°58′35″	107°03′08″	3,443
Avery	38°58′38″	106°58′44″	3,480

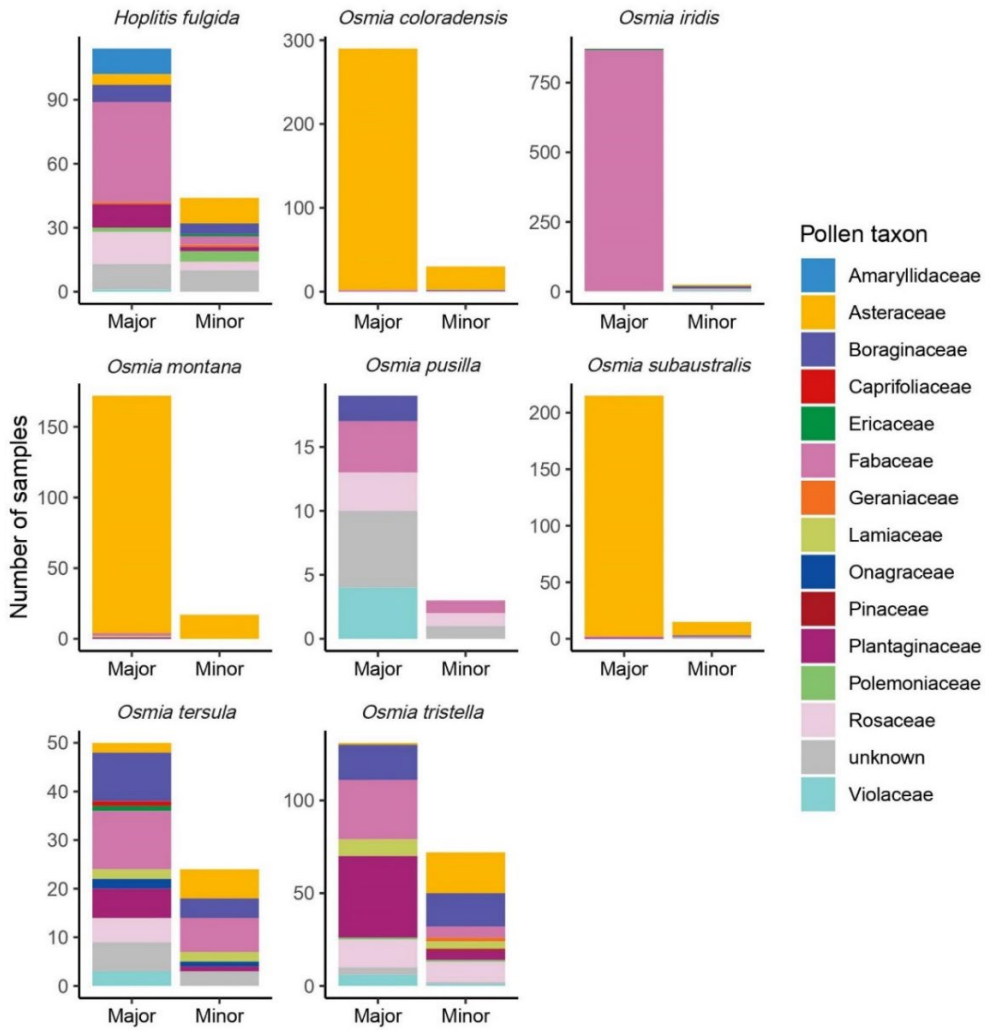

Figure 4-S1 Pollen usage of nine Osmiini species in our study area. The number of sampled brood provisions containing a given pollen family is presented for each Osmiini species and is separated between "major" and "minor" pollen components of the provisions. Each sample represents a small fraction of a complete pollen-and-nectar provision, collected as described by Spear et al. (2016). A pollen taxon was considered a major component if it represented more than 25% of the pollen in a provision, while pollen making up less than 25% of the provision was considered a minor component. The distinction between major and minor components was determined qualitatively, based on scanning the entire sample. Note that sample size was low for *Osmia pusilla* and we do not have data for *O. lignaria* at our study sites, but information on the pollen host-use of these species is available from previous studies (Wilson et al. 2010 and Roof et al. 2018 for *O. pusilla*, and Rust 1974, Barthell et al. 1997, and Haider et al. 2014 for *O. lignaria*).

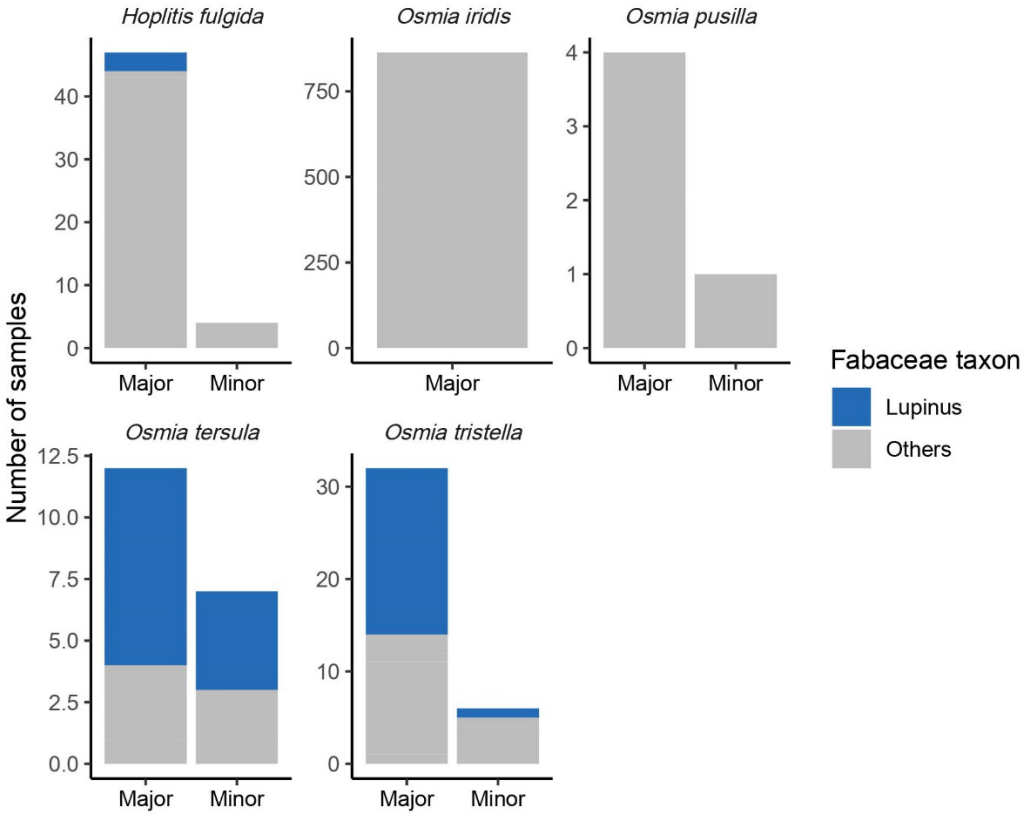

Figure 4-S2 Proportion of *Lupinus* and other Fabaceae taxa in the provisions containing Fabaceae pollen. The number of sampled provisions containing either *Lupinus* or other Fabaceae is presented for each Osmiini species and is separated between major and minor pollen components of the provisions (as defined in Fig. 4-S1). Sample size was low for *Osmia pusilla*, but information on the pollen host-use of this species is available from previous studies (Wilson et al. 2010, Roof et al. 2018).

Chapter 5: Do flower-colonizing microbes influence floral evolution? A test with fast-cycling Brassica

5.1 Abstract

Pollinators are thought to be the main drivers of floral evolution. Flowers are also colonized by abundant communities of microbes that can affect the interaction between plants and their pollinators. Very little is known, however, about how flower-colonizing microbes influence floral evolution. Here we performed an evolution experiment using fast-cycling *Brassica rapa* over six generations in which we manipulated the presence of pollinators and flower microbes to determine how pollinators and microbes interact in driving floral evolution. We measured the evolution of six plant traits, as well as plant mating system and flower attractiveness. Only one of the six traits evolved in response to pollinators, while microbes did not drive the evolution of any trait, nor did they interact with pollinators in driving floral evolution. Moreover, we did not find evidence that pollinators or microbes affected the evolution of flower attractiveness to pollinators. However, we found an interactive effect of pollinators and microbes on the evolution of autonomous selfing, a trait that is expected to evolve in response to pollinator limitation. Overall, we found only weak evidence that microbes mediate flower evolution. However, our ability to detect an interactive effect of pollinators and microbes might have been limited by weak pollinator-mediated selection in our experimental setting. Our results contrast with previous (similar) experimental evolution studies, highlighting the susceptibility of such experiments to drift and to experimental artefacts.

5.2 Introduction

Organisms are embedded in complex webs of interactions that affect species abundance, distribution, and evolution. In the last few decades, it has become clear that the outcome of mutualistic interactions is affected by the presence and abundance of other community members, such that these interactions cannot be fully understood in isolation from their community context. For example, herbivores can reduce the efficiency of pollination while mycorrhizal fungi can increase it (Strauss and Irwin 2004, Gange and Smith 2005, Agrawal et al. 2007, Afkhami et al. 2014, Bennett and Meek 2020). Much effort has recently been devoted to integrating the role of indirect effects of different community members in our understanding of the ecology of mutualisms (Chamberlain et al. 2014). Considering their ecological importance, indirect effects could also play an important role in the evolutionary trajectories of populations. However, we still know very little of how the evolution of mutualistic interactions is shaped by the surrounding community (Walsh 2013, Heckel and Kalisz 2017).

Animal pollination represents one of the key innovations of the flowering plants, having contributed to the impressive diversity of this clade (Van der Niet and Johnson 2012). Pollinators are thought to be the main drivers of floral evolution by selecting on flowers' attractiveness and efficiency at dispensing and receiving pollen (Harder and Johnson 2009). Yet, this mutually beneficial interaction is frequently exploited by antagonists and commensals (Irwin et al. 2003, Strauss and Whittall 2006, Ramos and Schiestl 2019). Because microorganisms are ubiquitous members of virtually every ecosystem, plants coexist with diverse microbial communities associated with their roots, vegetative tissues, and flowers (Trivedi et al. 2020). Despite their ephemeral nature, flowers are often colonized by numerous microorganisms, including nectar

specialists as well as generalist species associated with diverse plant organs (Manirajan et al. 2018, Russell and Ashman 2019, Vannette 2020). These microbes are not merely passive colonisers of the flower environment; flower-colonizing microbes can alter nectar sugar and amino acid composition, emit volatile compounds, and even warm flowers, thereby playing an integral role in shaping the flower phenotype (Herrera et al. 2008, Herrera and Pozo 2010, De Vega and Herrera 2013, Helletsgruber et al. 2017, Vannette and Fukami 2018, Rering et al. 2018, Schaeffer et al. 2019). In turn, these modifications of the flower phenotype can affect flower attractiveness to pollinators (Vannette et al. 2012, Rering et al. 2018, Schaeffer et al. 2019) and sometimes reduce or increase plant fitness (Vannette et al. 2012, Herrera et al. 2013, Schaeffer and Irwin 2014, Yang et al. 2019, de Vega et al. 2022, Tsuji 2023). Surprisingly, aside from plant pathogens (e.g., *Pseudomonas syringae*; Huang et al. 2012), there are few examples of direct impacts (i.e., impacts not mediated by pollinator attraction) of flower-colonizing microbes on plant fitness. One of the few instances was observed by Eisikowitch et al. (1990), who found that the yeast *Metschnikowia reukaufii* colonizing the nectar-filled stigmatic cavities of *Asclepias syriaca* reduces pollen germination.

Given that flowers are under selection by pollinators and that flower-colonizing microorganisms can influence pollinator behaviour (Francis et al. 2021), the floral microbiome could mediate flower evolution (Rebolleda-Gómez et al. 2019). For example, microbes that increase flower attractiveness to pollinators, such as some nectarivorous yeasts (Herrera et al. 2013, Schaeffer and Irwin 2014, Yang et al. 2019), could potentially select for floral traits favoring their growth, while pollinator-deterring microbes could select for antimicrobial properties. Indeed, it has been proposed that nectar and pollen secondary metabolites, as well as some volatile compounds, have evolved as antimicrobials (Adler 2000, Junker et al. 2011, Huang et al. 2012, Burdon et al.

2018, Boachon et al. 2019, Rivest and Forrest 2020). Similarly, flower colour, such as petal ultraviolet reflectance, has been shown to affect bacterial growth, and could thus be under selection by microbes (Rebolleda-Gómez et al. 2019, Hayes et al. 2021). Moreover, microbes could affect pollinator-mediated selection on floral scent, for example by increasing or decreasing the reliability of floral scent as an honest signal (Schaeffer and Irwin 2014). Finally, the evolution of flower sexual systems could be mediated by microbes (Rebolleda-Gómez et al. 2019), if, for example, microbes reduce the reliability or efficiency of pollinator services (Herrera et al. 2013), thereby increasing the need for self-fertilization. However, despite multiple potential influences of microbes in mediating selection on floral traits, very little is known about how flower-colonizing microbes affect floral evolution.

Determining the effect of the community context on the evolutionary trajectory of mutualistic interactions has been hindered by the long generation time of most organisms investigated, and most studies have been limited to predicting its outcome based on measures taken in a single generation (see Strauss and Whittall 2006 and references therein). Recently, experimental methods using a fast-growing strain of *Brassica rapa* have been developed, allowing experimental plant evolution in response to selection imposed by pollinators over a period of only ~ 2 years (Gervasi and Schiestl 2017, Ramos and Schiestl 2019). Here, we take advantage of this system by performing experimental evolution with plants exposed to either hand- or bumblebee-pollination and low or high microbial load for six generations to assess how pollinators and flower microbes interact in shaping floral evolution.

5.3 Methodology

5.3.1 Experimental design

We obtained seeds of fast-cycling *Brassica rapa* (Wisconsin Fast Plants™) with high genetic diversity (as specified by the supplier) from Merlan Scientific (Toronto, Canada). These plants have been shown to evolve in response to pollinator mediated selection in previous experimental evolution studies (Gervasi and Schiestl 2017, Ramos and Schiestl 2019), indicating that they exhibit sufficient genetic variation to be used in this type of study. These plants are partially self-incompatible, have a generalized pollination system, and have a generation time of approximately 35–40 days. The plants were crossed to produce 108 full-sib seed families (plants originating from a reciprocal cross between two parents) to constitute the starting populations (see Fig. 5-1).

For the first generation, plants were divided into four treatments: 1) hand pollination with low microbial load, 2) bumblebee pollination with low microbial load, 3) hand pollination with high microbial load, and 4) bumblebee pollination with high microbial load. Each treatment was divided into three replicates consisting of populations of 36 individuals (108 plants per treatment). Each plant family was used once per treatment and was represented in each treatment to control for genetic differences between treatments (i.e., replicate A of treatment 1 has the same family composition as replicate A of treatments 2, 3, and 4; see Fig 5-1). Plants were grown in individual pots ($7 \times 7 \times 8$ cm^3) using standardized soil (Pro-Mix, Canada) in growth chambers at 25°C, 50% humidity, and 24h of light (standard conditions for growing fast-cycling *Brassica rapa*), and were watered twice a day. Before sowing the plants of each generation, the soil was autoclaved, the growth chambers were cleaned with 1:10 bleach, and the seeds were sterilized with 70% ethanol for 1 minute to limit colonization of the plants by environmental microbes. The plants from each replicate and treatment evolved independently for six generations.

5.3.2 Microbial inoculation

The microorganisms used for the experiment were obtained from four wild populations of *Brassica rapa* collected in Ottawa, Canada, in semi-natural habitats (site coordinates: 45°28'03"N, 75°34'57"W; 45°24′46″N, 75°39′53″W; 45°24′47″N, 75°40′04″W; 45°25′02″N, 75°39′49″W). Flowers were collected from multiple individuals per population with sterile equipment using gloves. The flowers were brought to the laboratory, transferred to phosphate-buffered saline (PBS), sonicated for 10 minutes, and then vortexed for 10 minutes. The microbes were then plated on LB agar with cycloheximide for bacteria and yeast agar with chloramphenicol for yeasts. After incubation at room temperature for 5 days, the colony-forming units were counted and distinguished based on morphological attributes. We detected bacteria in 91% of the flower samples, while yeasts were present in 74% of the samples. The average bacterial abundance was $6.7 \times 10^4 \pm 2.7 \times 10^4$ per flower (mean ± SD, n = 68), and yeast abundance was $2.8 \times 10^4 \pm 8.7 \times 10^3$.

Six of the most common morphotypes of bacteria and one of the most common morphotypes of yeasts were selected for the experiment. The bacterial strains were identified by amplifying the 16S rRNA gene using primers 27F/1492R, while for the yeast strains we amplified the D1/D2 domain of the large subunit nuclear ribosomal RNA with primers NL1/NL4. The samples were sent for sequencing at Genome Québec and identified using Basic Local Alignment Search Tool (BLAST) searches against GenBank.

The yeast strain used for the experiment was identified as *Starmerella bombicola* (GenBank ID: OR344862). This species is closely associated with flowers and nectar as well as flower-visiting insects (primarily bees and beetles) (Rosa and Lachance 1998, De Graeve et al. 2018, Vannette

2020, Dolan et al. 2023). The bacterial strains we used were identified as *Acinetobacter* sp., *Brevundimonas* sp., *Microbacterium* sp., and *Pseudomonas* spp. (two strains) (GenBank IDs: OR334381-OR334385). These genera include multiple species associated with flowers and other plant organs, as well as bees (Mohr and Tebbe 2006, Junker et al. 2011, Aleklett et al. 2014, Burdon et al. 2018, Vannette 2020, Rathi and K N 2021, Alvarez-Perez et al. 2021) We were unable to identify one bacterial strain (using both the primers 27F/1492R and 515F/806R).

For each plant generation, we prepared a microbial inoculum by growing the bacteria and yeast strains overnight in liquid media. We removed the media by centrifuging and replacing the supernatant twice (replaced by 1:100 PBS in reverse-osmosis water for the first step and only reverse-osmosis water for the second step). We adjusted the concentration of each strain to a OD_{600} of 0.2 and mixed the strains in equal proportions to obtain a single inoculum (see Helletsgruber et al. 2017). The plants were inoculated using a nasal spray (fine mist atomizer with an injection volume of 0.15–0.2 ml) approximately 24 hours prior to pollination (18 days after sowing) with either the microbial inoculum (high-microbe treatments) or sterile distilled water (low-microbe treatments). The nasal spray was held at ~ 5 cm from the flowers and each open flower was sprayed once (only the inflorescences, not the leaves, were inoculated). We confirmed that the inoculation process resulted in relevant concentrations of microbes on flowers by inoculating, in addition to the experimental plants of the first generation, 16 plants with the microbial inoculum and 19 plants with a sterile inoculum and by isolating the microbes from the inoculated flowers 24h after inoculation (following the same procedure as above). The average bacterial and yeast abundances were $2.8 \times 10^5 \pm 2.8 \times 10^5$ and $6.8 \times 10^3 \pm 7.7 \times 10^3$ respectively in the high-microbe treatment, and $2.6 \times 10^2 \pm 4.4 \times 10^2$ and $2.2 \times 10^0 \pm 8.6 \times 10^0$ in the low-microbe treatment, implying that microbe abundances in the high-microbe experimental

treatments were, on average, approximately three orders of magnitude greater than those in the low-microbe treatments. The high variability in microbial abundance between flowers was consistent with what we observed in natural *Brassica rapa* populations (see above).

5.3.3 Pollination

We used bumblebees (*Bombus impatiens*) purchased from Biobest (Leamington, Canada) as pollinators for the experiment. We used one bumblebee colony per generation of *Brassica rapa* (colonies were purchased over a period of ~ 2 years). The colonies were supplemented with honeybee pollen and artificial nectar (provided by the supplier). Because bumblebees were inexperienced with flowers, we trained the bees on artificial flowers (Fig. 5-S1) in flight cages (the same as used for pollination; see below) for five days prior to pollination of the experimental *Brassica rapa*. Yellow artificial flowers rather than real *Brassica rapa* flowers were used during training to prevent bumblebees from associating reward with floral traits of plants with either low or high microbial loads (as microbes can affect the floral phenotype; see Introduction).

On day 19 after sowing, the plants were brought to flight cages of 2.5 m × 1.8 m × 1.2 m with standardized light conditions (artificial lighting producing visible and ultraviolet light). Plants were randomly placed in a 6 × 6 array. For the bumblebee-pollination treatments, the bees were starved 16h before the onset of pollination. Three to five bumblebees were then released individually in a flight cage and allowed to visit up to five different plants. Each bee was used only once. Twelve to 15 plants were pollinated per replicate. The open flowers from the visited plants were marked and only seeds from these flowers were collected. Pollination of only a subset of the 36 plants per replicate allowed us to simulate pollinator limitation.

For the hand-pollination treatments, 12 of the 36 plants per replicate were randomly selected to be pollinated. For each of the 12 plants, three flowers (because some plants produced few flowers, and hand-pollinated flowers typically had high seed production) were hand pollinated using a metal probe with pollen from another randomly selected plant among the same group of 12 plants (the pollen donors could be used more than once) (Gervasi and Schiestl 2017, Ramos and Schiestl 2019). The hand-pollinated flowers were marked and only seeds from these flowers were collected. Hand-pollinating a randomly selected subset of plants (12 of the 36 plants per replicate) controlled for the genetic bottleneck produced in the bumblebee-pollination treatments (by allowing only a subset of plants to be pollinated), while removing any selection by pollinators on floral traits (as plants were selected randomly).

5.3.4 Seed collection

After pollination was completed, the plants were brought back to growth chambers. Approximately 30 days after pollination, seeds were collected from the marked flowers of the pollinated plants. We counted the number of seeds per plant and quantified their (maternal) relative fitness as the individual's number of seeds produced divided by the mean seed set of the plants from the same replicate. Each plant contributed to the next generation proportionally to their relative fitness, with plants producing more seeds contributing more to the next generation. More specifically, the number of seeds that each plant contributed to the next generation was calculated as 36/(total number of seeds of the replicate/individual seed set) (see Gervasi and Schiestl 2017, Ramos and Schiestl 2019).

5.3.5 Inter-replicate crossing

After the six generations of experimental evolution, we grew the plants for two generations without microbes or pollinators. Plants of generation 7 were crossed between replicates within treatments to produce generation 8. This was done by growing seeds produced by generation 6 and hand-pollinating the plants with donors from different replicates within each treatment, with each replicate serving both as pollen donor and receiver once (see Gervasi and Schiestl 2017, Ramos and Schiestl 2019). This allowed us to avoid potential maternal effects within treatment (e.g., due to flower-colonizing microbes) and to reduce the effect of inbreeding on plant traits by restoring heterozygosity (as fewer than half the plants in each generation contributed to the next generation; Gervasi and Schiestl 2017, Ramos and Schiestl 2019). However, because of the inter-replicate crossing, replicates within treatment were no longer genetically independent at generation 8. We therefore analysed plant traits at both generations 7 and 8 to overcome the limitations in interpreting results from each generation.

5.3.6 Measurement of plant traits

For generations 1, 7, and 8, approximately 20–25 plants per replicate per treatment were randomly selected for the measurement of morphological traits. Traits were measured 19–21 days after sowing. We counted the total number of flowers, including withered flowers and unopened buds (to avoid having the flower count be influenced by the day on which the measures were taken). We measured plant height with a ruler. Pistil length, stamen length, and petal length were measured on three haphazardly selected fully opened flowers per plant (avoiding terminal flowers, flowers showing signs of withering, and atypical flowers) using a digital caliper. We measured the total area and the ultraviolet-absorbing area of the four petals of one haphazardly selected flower per plant. The petals were first removed and placed between

two microscope slides on top of a white sheet of paper. We took photographs of the petals with a Nikon D70 camera mounted with a Baader U-Filter (Baader Planetarium, Mammendorf, Germany), which allows the passage of ultraviolet light while blocking visible light (bandwidth = 320–380nm). The total area and the ultraviolet-absorbing area of the petals were then measured using the software ImageJ (Schneider et al. 2012). These plant traits were selected because they were measured in previous experimental evolution studies with *B. rapa* and were found to evolve in response to pollinators (Gervasi and Schiestl 2017, Ramos and Schiestl 2019; see also Conner and Rush 1996, Donnelly et al. 1998, Thompson 2001, Chen et al. 2020, Rowe et al. 2020, for the role of the traits we investigated in pollinator attraction).

5.3.7 Autonomous selfing

To test for autonomous selfing, we grew approximately 20 plants per replicate per treatment from generation 8 (from a different set of plants than for the measurement of plant traits). The plants were not manipulated or moved during their flowering period. Approximately 30 days after peak flowering, we counted the number of fruits and the number of seed per fruit (on all the fruits produced per plant) as measures of autonomous selfing.

5.3.8 Bee choice assays

We tested bumblebee preferences among plants of the four treatments from generation 8. The bees used for the assays were trained in the same way as for experimental evolution (see above). The choice assays were performed 19–20 days after sowing. We placed one plant of each treatment in a flight cage (2.5 m × 1.8 m × 1.2 m) in a square configuration with one plant on each corner and approximately 25 cm between plants (Gervasi and Schiestl 2017, Ramos and Schiestl 2019). During each individual assay, one bee was released and allowed to visit only one

plant. We considered a visit to have occurred only when the bee landed on an inflorescence and collected nectar or pollen. We recorded the number of open flowers and the position of the plants. Each plant and each bee were used for a single assay (to ensure complete independence between assays) and the positions of the treatments in the flight cages were rotated counterclockwise between assays. In total we performed 80 assays (with 320 plants and 80 bees from four colonies; the colonies were different from the ones used for experimental evolution).

5.3.9 Statistical analysis

Effects of pollinators, microbes, and their interaction

We used Bayesian models with normal distributions to test whether pollinators and microbes influence floral evolution and whether these potential agents of selection interact in driving floral evolution. Using Bayesian models allowed us to test the effect of pollinators, microbes, and their interaction on multiple traits without introducing problems of multiple testing. We first measured changes in trait values (for plant height, number of flowers, pistil length, stamen length, petal length, and proportion of ultraviolet-absorbing petal area) between generation 1 and both generations 7 and 8 by subtracting the replicate-by-treatment mean of the trait value at generation 1 from the values at either generation 7 or 8. We fitted models of changes in trait values for generations 7 and 8 (including both generations in the same model for each trait). We used pollinator treatment, microbe treatment, and generation as explanatory variables, as well as all possible interactions between pollinators, microbes, and generation. We incorporated replicate (three levels) as well as all possible interactions between pollinators, microbes, and replicate as random variables.

We interpreted a significant interaction between generation and microbes, pollinators, or microbes × pollinators as inconsistent evolutionary trajectories before and after inter-replicate crossing (i.e., generation 7 vs. 8), while the absence of such interactions indicated that the evolutionary trajectories were consistent. We interpreted an overall effect of pollinators, microbes, or their interaction as evidence of selection, while a significant interaction between replicate and these variables was interpreted as evidence of evolutionary drift. We tested the significance of random interactions between replicate and pollinators, microbes, and the interaction between pollinators and microbes by comparing models with and without these random variables using leave-one-out cross-validation (with the function loo from the R package loo; Vehtari et al. 2016).

We used non-informative priors for all parameter estimates. The models were run using Hamiltonian Monte Carlo sampling, with four chains of 4,000 iterations, of which the first 1000 were discarded as burn-in. The thinning intervals were set to 8 to reduce autocorrelation in the Markov chains. Convergence of chains for all parameters was verified both visually with trace plots and with the Gelman–Rubin convergence statistic (< 1.01 for all parameters) (Gelman and Rubin 1992). The models were run using the brm function from the brms package in R (Bürkner 2017).

We looked at correlations among plant traits to determine whether the evolution of some traits could be the result of correlated responses rather than independent evolution. We conducted all analyses in R version 4.1.1. (R Core Team, 2020).

Mating systems

Change in herkogamy (the distance between stigmas and anthers) was evaluated in the same way as change in other plant traits (above).

The effects of pollinators and microbes on the proportion of autonomously selfing plants at generation 8 were evaluated using a Bernoulli distribution and a logit link, with the same explanatory and random variables as for the trait models, except for generation and its interactions, which were absent from this model. We used the same Bayesian approach as for the trait analysis.

Bee choice assays

We tested the effect of pollinators and microbes on bumblebees' preferences for plants at generation 8 using a Bernoulli distribution and a logit link. Bee choice, coded as 1 for visited plants and 0 for non-visited plants, was used as the response variable. We incorporated the same explanatory and random variables as in the model of autonomous selfing (above), as well as number of flowers coded as a fixed variable, and position in the flight cage (one of the four corners) and bee colony (as more than one colony was used for the bee choice assays) coded as random variables. We used the same Bayesian approach as for the analysis of floral traits and mating systems.

5.4 Results

Overall, few plant traits showed an evolutionary response to pollinators. Plants exposed to pollinators evolved greater flower production than hand-pollinated plants (Fig. 5-2F; Bayesian credible interval (CI) of the effect of pollinators on flower number = 0.15, 6.23; n = 634), but other traits were unaffected (i.e., the credible interval overlapped zero; see Table 5-S1, Fig. 5-2).

Microbes did not affect the evolution of floral traits, nor did they interact with pollinators in driving floral trait evolution (Fig. 5-2, Table 5-S1).

Similarly, changes in herkogamy (the distance between stigmas and anthers) did not differ much among treatments (Fig. 5-3A, Table 5-S1). There was an interaction between pollinators and microbes in determining the proportion of plants exhibiting autonomous selfing at generation 8 (Fig. 5-3B; CI = 0.08, 2.88, n = 236), although we did not find effects of pollinators or microbes alone (CI = −1.31, 1.41 for pollinators, and −2.15, 0.23 for microbes). There was no noticeable difference in selfing ability between plants exposed to low or high microbial loads in bumblebee-pollinated plants; however, hand-pollinated plants exposed to low microbial loads had the highest selfing ability, while plants from the hand-pollinated–high-microbial-loads treatment had the lowest.

Although bumblebee-pollinated plants evolved larger floral displays than hand-pollinated plants, bees did not prefer plants from the bumblebee-pollinated treatments at generation 8 (Fig. 5-4, CI of the preference for the bumblebee-pollinated treatments = −1.00, 1.69; n = 320 plants in 80 assays). Similarly, bees did not distinguish between plants that evolved in low- or high-microbe conditions (CI of the effect of microbes = −1.81, 0.71). However, we detected a preference for plants with more flowers in the choice assays, consistent with the evolution of larger floral displays in bumblebee-pollinated plants (CI = 0.04, 0.45).

Some plant traits were correlated with each other, but strong correlations were observed only for a few traits. Specifically, we found strong correlations between plant height and flower number (r = 0.62), plant height and pistil length (r = 0.49), and petal and stamen length (r = 0.61). However, since we only found evidence of divergent evolution between treatments in the

number of flowers, correlated evolutionary responses among floral traits should not impact our conclusions.

Evolutionary changes in plant traits often varied among replicates within treatments, consistent with evolutionary drift rather than selection (Fig. 5-S2). Indeed, the incorporation of a random interaction between replicate and treatment (pollinators, microbes, and pollinators × microbes) improved model fits for all traits expect pistil length.

We found some inconsistencies between generations 7 and 8 (i.e., before and after inter-replicate crossing), evidenced by interactions between generation and the pollinator or microbe treatments (for number of flowers, plant height, and UV-absorbing area). This indicates that some evolutionary changes differed before and after inter-replicate crossing. We found such an interaction for the evolution of flower number, for which we also detected an effect of pollinators. However, the patterns of flower number between treatments were consistent between generations, suggesting that only the strength of the difference between treatments, rather than its direction, varied between generations 7 and 8.

5.5 Discussion

Many floral traits, such as flower size, colour, and scent, are undeniably the result of selection exerted by pollinators (Fenster et al. 2004, Raguso 2008, Harder and Johnson 2009). Yet, flowers are also colonized by numerous taxa of bacteria and fungi that can mediate the interaction between plants and their pollinators, but their role in floral evolution remains mostly unknown (Rebolleda-Gómez et al. 2019, Vannette 2020). We found little evidence that pollinators and microbes interact in driving floral evolution in our experimental evolution study. Microbes did not drive the evolution of any of the plant traits we measured, nor did they

influence evolutionary responses to pollinators (Fig. 5-2). This being said, there was little response even to pollinator-mediated selection in our experimental setting. Indeed, bumblebee-pollinated plants produced more flowers relative to hand-pollinated plants after experimental evolution, but exposure to pollinators did not influence any of the other five plant traits that we measured (petal, stamen and pistil length, ultraviolet absorbing area, and plant height; Fig. 5-2). Our ability to detect interactions between pollinators and microbes was therefore limited. Pollinators are known to be important agents of selection on many floral traits in the wild (Fenster et al. 2004, Raguso 2008, Harder and Johnson 2009), and other experimental evolution studies have observed evolutionary trajectories consistent with the pollinator-mediated selection observed in nature (Gervasi and Schiestl 2017, Ramos and Schiestl 2019). Because of this inconsistency between our study and other experimental evolution studies, as well as many natural populations, our results should be interpreted with care; microbes could influence pollinator-driven evolution of floral traits for which we did not detect evolutionary changes in response to pollinators. Nevertheless, our results suggest that microbes are not likely to be strong drivers of floral evolution on their own—that is, as direct drivers of floral evolution rather then by mediating pollinator-driven evolution. Indeed, microbes alone did not affect the evolution of the six plant traits that we investigated, although they appeared to influence mating system evolution.

Flower-colonizing microbes might impact the evolution of plant mating systems. Indeed, self-pollination is thought to evolve in response to limited or inconsistent pollinator availability (Lloyd 1979, Larson and Barrett 2000), and bacteria could increase or decrease the need for self-pollination, for example, by affecting flower attractiveness to pollinators (Rebolleda-Gómez et al. 2019). We found some support for an interactive role of microbes and pollinators in driving

the evolution of autonomous selfing. Although in bumblebee-pollinated plants selfing ability was similar between plants exposed to a low or high microbial load, in hand-pollinated plants, exposure to a low microbial load resulted in considerably lower selfing ability relative to plants exposed to a high microbial load (Fig. 5-3B). A few studies have found that nectar-inhabiting microbes can induce pollen germination in pollen grains that have fallen into nectar (Eisikowitch et al. 1990, Eisdcowitch et al. 1990, Christensen et al. 2021), raising questions about whether similar mechanisms could occur on flower stigmas (Cullen et al. 2021). We could imagine that, similar to what previous studies have reported for nectar-specialist bacteria and yeasts, microbes colonizing the stigmas of *Brassica rapa* could facilitate pollen germination, and hence reduce selection for autonomous selfing. However, the higher selfing ability that evolved in hand-pollinated plants with low microbial loads is hard to reconcile with the fact that these plants likely received far more pollen than bumblebee-pollinated plants. Hand pollination often results in the application of an excess of pollen relative to animal pollination (Ashman et al. 2004). We would therefore have expected plants pollinated by bumblebees to evolve *higher* selfing rates, because seed set should have been more limited by pollen receipt. It is hard to understand why in low-microbe settings hand-pollinated plants would evolve higher selfing abilities than bumblebee-pollinated plants (although in high-microbe conditions the evolution of lower selfing ability in the hand- vs. bumblebee-pollinated plants matches our expectations).

It is possible that the interaction we found between pollinators and microbes in driving the evolution of selfing rate represents a spurious finding. For example, although our model controlled for the potential for evolutionary drift among replicates to drive the evolution of floral traits, we would interpret such drift as selection if drift occurred in the same direction in all three replicates of a given treatment. Considering that we performed multiple tests, it seems plausible

that this scenario occurred at least once (even though the Bayesian framework that we used for our statistical analyses avoids problems of multiple testing, it does not allow us to differentiate between drift and selection in this scenario).

Overall, we found a stronger role of evolutionary drift than selection in shaping the evolution of floral traits. Consistent changes among replicates within treatments would have suggested a primary role of selection, while we often found considerable variation among replicates, which is more consistent with drift (Fig. 5-S2). Evolutionary drift is accentuated in small populations, for which random changes in allele frequencies can play a predominant role in the trajectories of the populations' genotypes. With 36 plants per replicate per treatment, the size of our populations was relatively small (but the same as in previous experimental evolution studies; Gervasi and Schiestl 2017, Ramos and Schiestl 2019). Moreover, only approximately half of the plants were pollinated in a given generation, which contributed to reducing the effective population size of our experimental populations. While this approach allowed us to potentially produce strong pollinator-mediated selection, it also meant that strong selection would have been necessary to counteract drift. In our study, it seems apparent that, for most traits, selection was not strong enough to counter evolutionary drift.

Pollinators can act as strong agents of selection on floral traits, especially when access to pollinators is limited. By simulating this scenario, previous experimental evolution studies found that plants evolved phenotypes associated with higher attractiveness to pollinators (Gervasi and Schiestl 2017, Ramos and Schiestl 2019). In our study, however, only a single floral trait evolved in this direction (flower number). Our approach mostly replicated the methodology used in previous experimental evolution studies which also used fast-cycling *Brassica rapa* (Gervasi

and Schiestl 2017, Ramos and Schiestl 2019), but differed in a few key aspects that could explain the discrepancies in floral evolution between studies. First, in contrast to previous studies, we did not expose bumblebees to real *Brassica rapa* flowers during training (see Methodology). Because pollinators can associate floral characteristics with reward, including the presence or absence of microbes (Russell and Ashman 2019), using real flowers for training could have led us to detect flower evolution that was driven by associative learning for the microbial load of the plants used during training, rather than by an ecologically relevant scenario. The lack of training on real *Brassica* flowers, however, might have prevented bumblebees from selecting for floral traits associated with higher reward production (i.e., honest signals; Knauer and Schiestl 2015), because bees could not learn to associate reward production with traits correlated with reward (as was suggested to occur in a previous experimental evolution study; Gervasi and Schiestl 2017). Another factor that might have played a role is the use of different pollinator species: *Bombus impatiens* in our study, vs. *B. terrestris* in previous studies. The native range of *Brassica rapa* overlaps with the range of *B. terrestris*, but not with that of *B. impatiens*. Because of their shared evolutionary history*, Br. rapa* might respond more to selection driven by *B. terrestris* (although *Br. rapa* has a generalist pollination syndrome, and *Br. rapa* and *B. impatiens* have co-occurred in North America for many decades).

These discrepancies between our study and previous experimental evolution studies highlight the sensitivity of the conclusions of such studies to the specific laboratory conditions. While our results do not invalidate previous findings, they point to the importance of carefully designing such experiments to adequately mimic natural scenarios. For example, the use of artificial flowers during training allowed us to avoid spurious findings due to bees associating a certain microbial condition with reward (as was found by Russell and Ashman, 2019), but at the

potential cost of reduced pollinator-mediated selection. Overall, the lack of realism in experimental evolution studies, for example due to strong simulated pollinator limitation (which may not mimic typical natural conditions) and pollination in highly controlled laboratory conditions, might limit the extant to which we can draw conclusions about natural systems from such studies. These experiments should therefore be interpreted as proof-of-concept studies, hinting at the potential for processes to occur or not in nature, rather than as direct evidence for such processes.

To our knowledge, our study represents the first experimental test of the role of microbes in mediating floral evolution. In order to isolate the role of microbes and pollinators, and to observe flower evolution in real time, we conducted our experiment in controlled laboratory conditions. Our findings might therefore differ considerably from what happens in natural settings. For example, interactions with microbes and pollinators can vary among and within plant populations, resulting in substantial variation in selection exerted by pollinators, and, potentially, microbes (Ashman et al. 2004, Knight et al. 2005, Francis et al. 2023). Moreover, we manually inoculated our experimental plants, while microbial dispersal is partly carried out by pollinators in the wild, which could influence the interplay between pollinators and microbes in driving floral evolution (Russell et al. 2019, Keller et al. 2021, Francis et al. 2023). Another important caveat is that the use of culture-dependant methods to grow our microbes likely biased our bacterial community towards generalist species (although the yeast species we used in the experiment is a flower specialist; see Methodology). Finally, in order to simplify our experimental system, we did not allow microbes to evolve in response to flower evolution, although in natural conditions microbes and plants (and pollinators) likely co-evolve in response to changes in their interaction partners' phenotypes (Rebolleda-Gómez et al. 2019).

Our study therefore represents a first proof-of-concept experiment, showing limited support for a role of microbes in flower evolution. Despite our largely negative results, we believe that more studies are needed that integrate microbial ecology in the study of flower evolution, given that the interaction between plants, pollinators, and microbes is likely to vary considerably with ecological context. In particular, studies are needed that investigate the role of microbes in mediating selection on various floral traits, how floral traits drive the evolution of microbes, and ultimately, how plants, pollinators, and microbes co-evolve in complex communities. The integration of microbes into pollination ecology is still in its infancy, yet it is becoming more and more evident that they are intimately intertwined in plant–pollinator interactions (Vannette 2020, Francis et al. 2021, Keller et al. 2021). Determining when and how microbes affect the ecology and evolution of pollination promises to improve our understanding of this fascinating and complex interaction.

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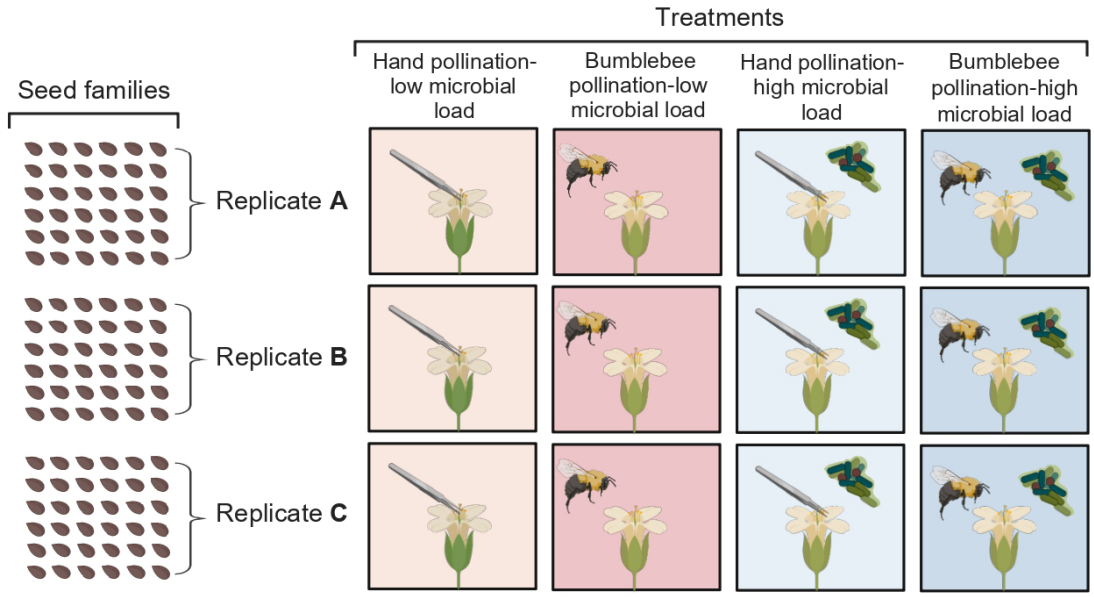

Figure 5-1 Design of our experimental evolution study. 108 full-sib families of *Brassica rapa* were separated into three replicates (36 plants each), with each family being represented in each treatment.

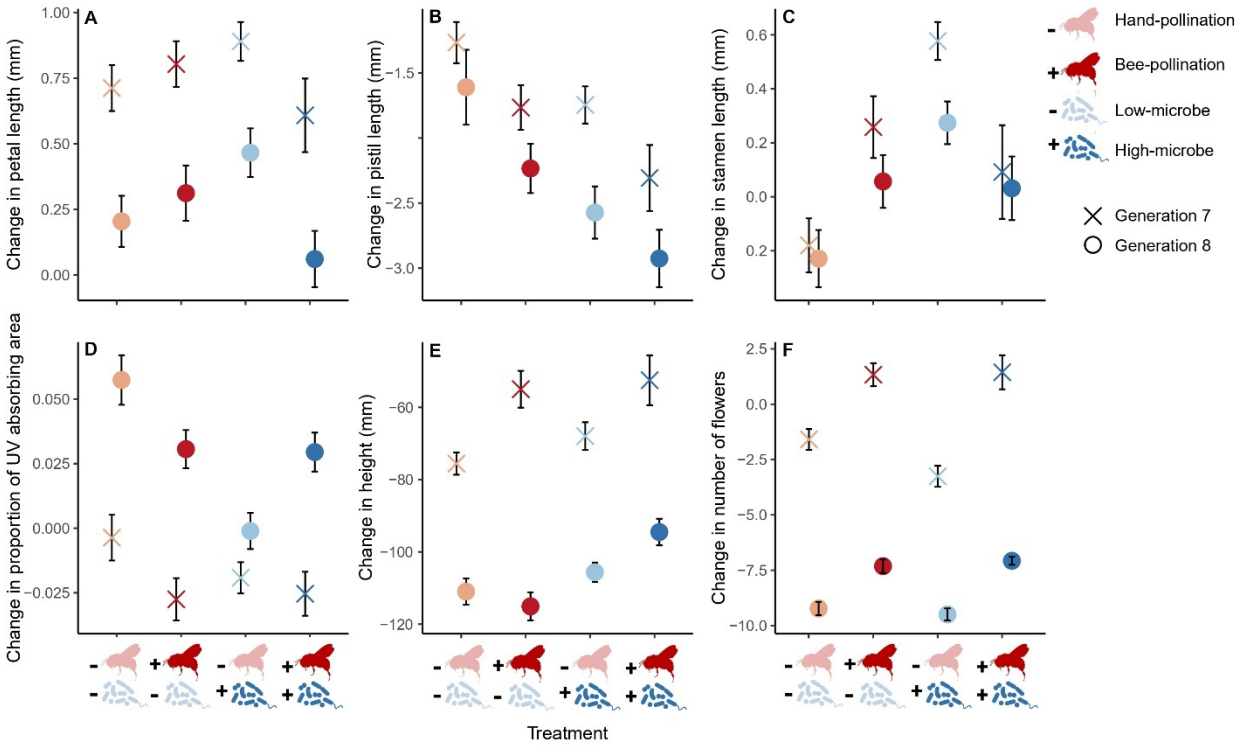

Figure 5-2 Change in plant traits between generation 1 and generations 7 (before inter-replicate crossing) and 8 (after inter-replicate crossing) in four treatments: hand-pollination and low microbial load, bumblebee-pollination and low microbial load, hand-pollination and high microbial load, and bumblebee-pollination and high microbial load, for petal length (A), pistil length (B), stamen length (C), proportion of ultraviolet-absorbing area (D), plant height (E), and number of flowers (F). We detected an effect of pollinators only on flower number. No effect of microbes or the interaction between pollinators and microbes were detected for any of the six traits. The error bars represent standard errors.

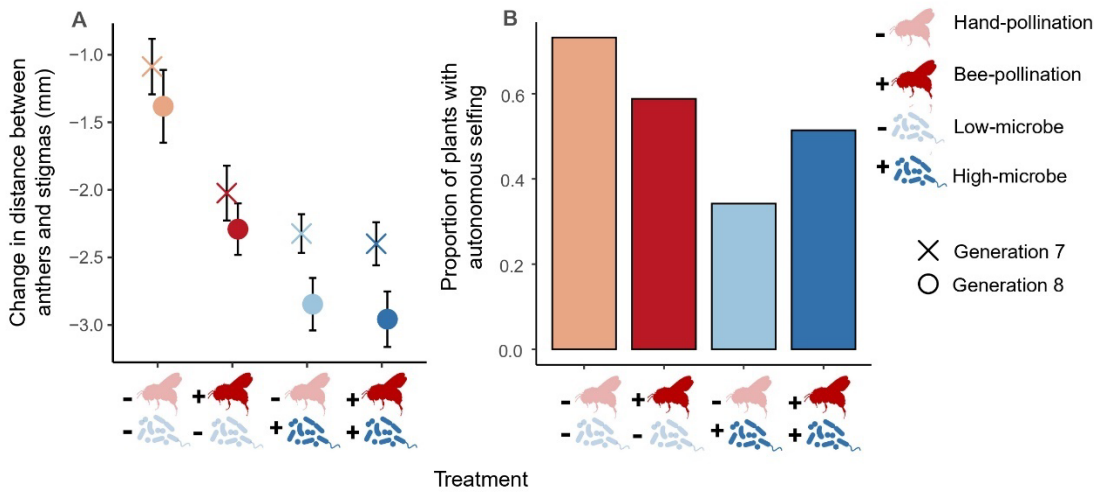

Figure 5-3 Effects of pollinators, microbes, and their interaction on the evolution of mating systems in *Brassica rapa*. Change in herkogamy between generation 1 and generations 7 (before inter-replicate crossing) and 8 (after inter-replicate crossing) in four treatments: hand-pollination and low microbial load, bumblebee-pollination and low microbial load, hand-pollination and high microbial load, and bumblebee-pollination and high microbial load (A). Proportion of plants producing seeds by autonomous selfing at generation 8 in the four treatments (B).

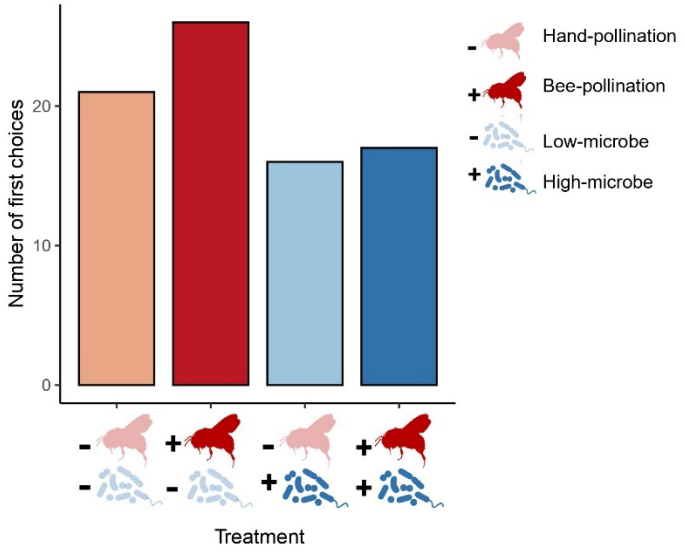

Figure 5-4 Bumblebee preference for plants of generation 8 in four treatments: hand-pollination and low microbial load, bumblebee-pollination and low microbial load, hand-pollination and high microbial load, and bumblebee-pollination and high microbial load.

Table 5-S1 Effect of pollinators, microbes, and their interaction on the evolution of six floral traits between generation 1 and generations 7 and 8 (pooled in the same models). An effect is considered significant when the credible interval of the estimated effect size does not overlap zero.

Floral trait	Sample size	Factor	Credible interval
Petal length	634	Pollinator	-1.43, 1.17
		Microbe	-1.13, 0.90
		Pollinator × microbe	-1.32, 0.12
Stamen length	634	Pollinator	-4.47, 4.45
		Microbe	-0.99, 1.48
		Pollinator × microbe	-2.19, 0.49
Pistil length	634	Pollinator	-1.03, 0.60
		Microbe	-2.03, 0.68
		Pollinator × microbe	-1.07, 1.05
Proportion UV-absorbing area	500	Pollinator	-0.10, 0.07
		Microbe	-0.11, 0.08
		Pollinator × microbe	-0.05, 0.12
Plant height	634	Pollinator	-2.31, 4.71
		Microbe	-2.48, 4.38
		Pollinator × microbe	-3.60, 3.96
Number of flowers	634	Pollinator	0.15, 6.23
		Microbe	-4.45, 3.91
		Pollinator × microbe	-1.33, 4.76

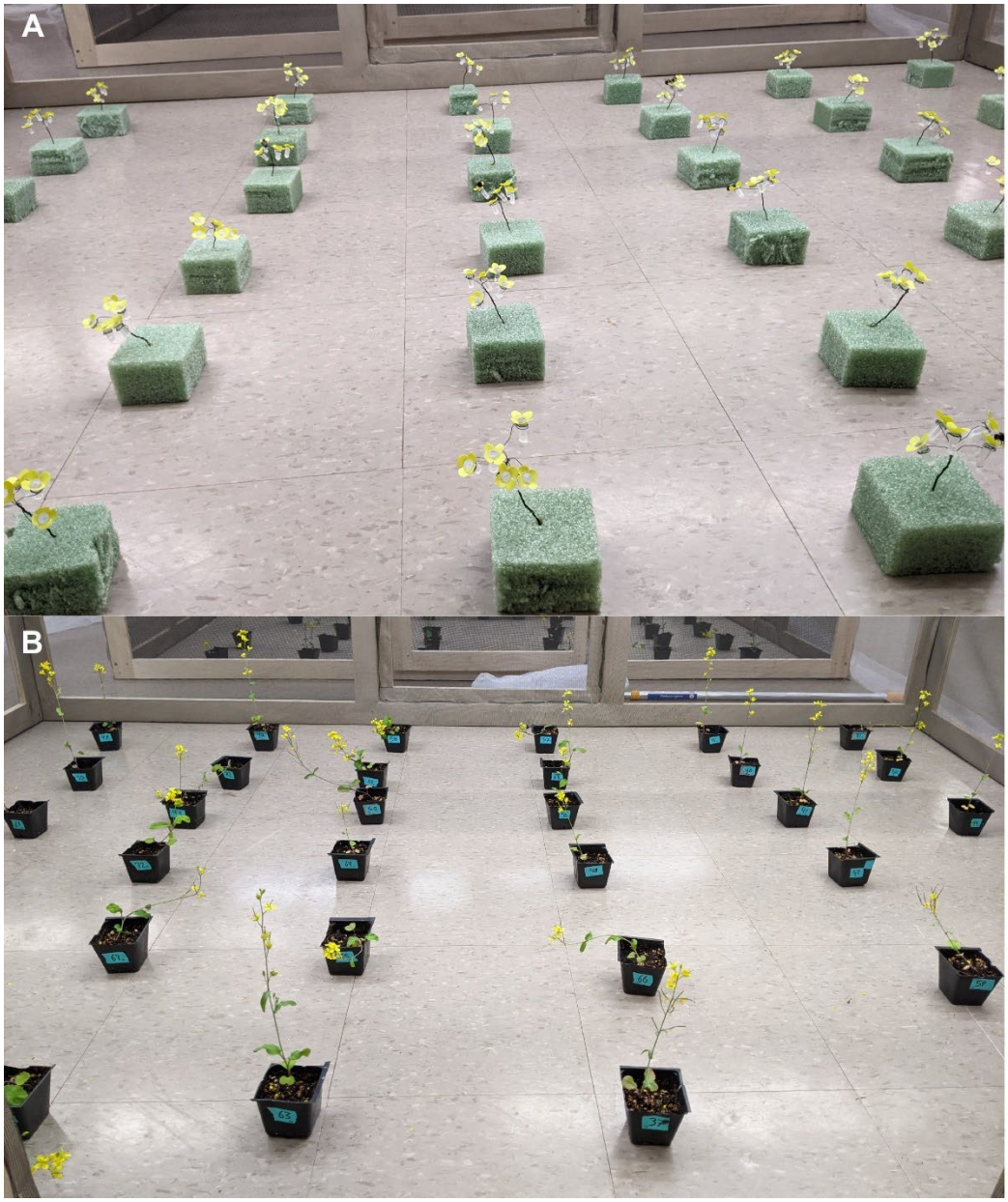

Figure 5-S1 Bumblebee training and pollination of *Brassica rapa* in flight cages. Artificial flowers used during the training of the bumblebee colonies used for the pollination of *B. rapa* during experimental evolution (A). *Brassica rapa* flowers disposed in the flight cage for pollination (B).

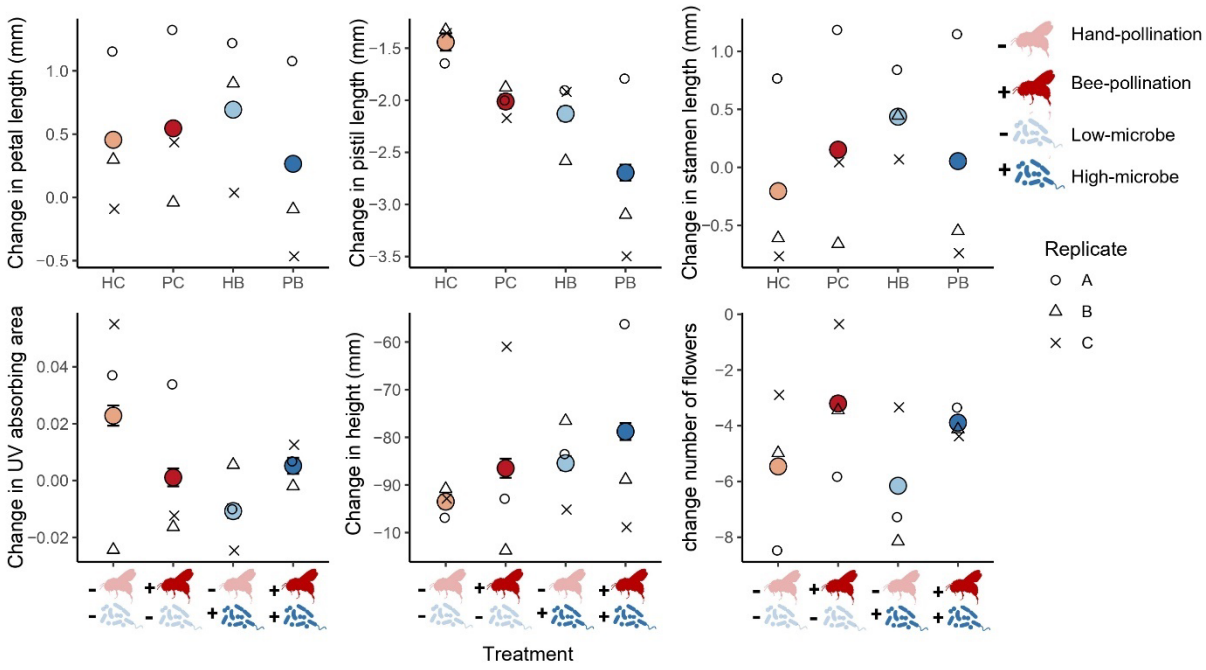

Figure 5-S2 Change in floral traits between generation 1 and generations 7 and 8 (pooled) for each replicate in four treatments: hand-pollination and low microbial load, bumblebee-pollination and low microbial load, hand-pollination and high microbial load, and bumblebee-pollination and high microbial load, for petal length (A), pistil length (B), stamen length (C), proportion of ultraviolet absorbing area (D), plant height (E), and number of flowers (F). The error bars represent standard errors.

Chapter 6: General discussion

Pollination has traditionally been perceived as an intimate and mutually beneficial relationship between plants and their pollinators (Waser et al. 1996). Yet, the presence of defensive compounds and spines in the pollen of diverse plant families seems paradoxical in this context: why do plants seem to poison their pollinators, and how do these apparent defences affect pollination? I used the word "seems" before "paradoxical" here and in previous chapters because, as I argue in this thesis, the existence of defensive floral traits is paradoxical only in the narrow view of pollination as a tight mutualism between plants and pollinators. I also argue that pollen defences—an antagonistic component in a *mostly* mutualistic interaction—could result in surprising similarities in evolutionary patterns between pollination and herbivory (an antagonistic interaction). In bringing the chapters of this thesis together, one fascinating implication is that some plant antagonists might play a major role in the evolution of pollination.

In Chapter 2, I aggregated the scientific literature on occurrences of defence compounds in pollen and I laid out hypotheses to explain their presence. No effort had been previously made to assemble and organize knowledge and hypotheses regarding pollen defences into a cohesive research program. However, such an effort had been made for toxic nectar by Adler (2000) more than 20 years ago, and proved an effective catalyst for the study of nectar secondary metabolites. One major finding from Chapter 2 is that pollen defence compounds are relatively widespread across plant families. Still, even since this review has been published three years ago, multiple new instances of pollen secondary metabolites with known toxic effects on insects have been uncovered (Trunz et al. 2020, Ritmejerytė et al. 2020, 2023, Cane et al. 2020, Li et al. 2022). It is

likely that the proportion of plants with defensive compounds in their pollen is greatly underestimated. One likely reason for this is that the realisation that floral rewards can contain such compounds is relatively new. Challenges with sampling and analysing small quantities of floral rewards probably also contributed to slowing progress in the study of floral reward chemistry. Pollen has been scanned for its chemical composition in only a small fraction of plant genera, and in considerably fewer species than for vegetative tissues. Mapping the chemical composition of pollen across plant taxa as has been done for vegetative tissues is a distant goal, but as findings from my thesis suggest, it could prove valuable in furthering our understanding of pollination ecology.

6.1 Causes of pollen defences

Given that pollen defences appear to be common among flowering plants (Chapter 2), the logical next step was to determine the causes of this floral trait. In Chapter 3, I aimed at testing the hypotheses that I proposed in Chapter 2 to explain the presence of pollen defences: the pleiotropy hypothesis, the protection-against-pollen-collection-hypothesis, and the antimicrobial hypothesis. Because they are not mutually exclusive, one important strength of the study was that I tested the three hypotheses simultaneously (by studying a single hypothesis, one might conclude that one had found the cause of the trait while still missing significant parts of the story). This being said, I only tested hypotheses that seemed probable in light of our current knowledge of pollination ecology, and I might have myself missed important parts of the story. With this in mind, I was able to demonstrate that pollinators are unlikely to be the main drivers of pollen defences in *Lupinus argenteus*. More likely, pollen defences partly originate from "spillover" from adjacent tissues heavily protected against antagonists. I also found evidence that pollen defence compounds might mediate the interaction between pollen and bacteria by

reducing bacterial colonization of pollen. It is however too early to confidently tell whether this potential mediating role is adaptive or simply a byproduct of defence compounds often being detrimental to both herbivores and microorganisms (see Chapter 2). The role of microorganisms in pollination is still a field of research in its infancy, and the exact fitness consequences of pollen-colonizing microbes remain untested (Vannette 2020). Another interesting finding from this chapter is that pollinators might not select for lower concentrations of pollen defensive compounds, expect perhaps at very high concentrations. This could allow the maintenance of defensive compounds in pollen even if there is no strong selective pressure for their maintenance in this reproductive tissue. This last finding provides a simple answer to the apparent paradox of chemically defended pollen: bees are not deterred by naturally occurring concentrations of the pollen secondary metabolites of their hosts. This can explain the apparent contradiction between the presence of alkaloids in *Lupinus* pollen and the hypothesis that plants with concealed pollen or that depend on pollen-collecting bees should not have defended pollen (Sedivy et al. 2011, Trunz et al. 2020).

My work in Chapter 3 only involved a single plant species, and I expect that the causes for the presence of pollen defences vary considerably between species. Overall, available evidence across studies provides some support for each of the three hypotheses that I proposed in Chapter 2. Of the few studies conducted to date that have looked at the correlation in defence compound concentrations between pollen and other tissues, all found some positive correlations (Kessler and Halitschke 2009, Heiling et al. 2019, Trunz et al. 2020, Chapter 3 of this thesis). Virtually every plant species possesses some kind of chemical defence in their vegetative tissues. The potential for these defences to occur in other tissues as well, whether due to physiological spillover or pleiotropy, might be widespread. Whether plants experience selection to increase or

decrease secondary metabolite concentration from this "baseline" might be highly context-dependent, varying as a function of the mode of pollination (for example, bees might select for deterrent pollen in nectar-rewarding plants; Wang et al. 2019), and the local community context. For example, in the Colorado Rocky Mountains, *Lupinus argenteus* shows variable but relatively high concentrations of alkaloids in its pollen, while no alkaloids have been detected in the pollen of *L. bakerii* (Heiling et al. 2019), despite the fact that the two species exhibit some overlap in distribution, both species produce high concentrations of alkaloids in their flowers and vegetative tissues, and both are pollinated by mostly the same insects (personal observation). The cause of this interspecific difference remains unclear, but solving these kinds of discrepancies could be key in understanding the evolution of pollen defences. Phylogenetic studies, in particular, could be a valuable tool in deciphering the conditions driving the evolution of stronger or weaker pollen chemical defences (see Trunz et al. 2020, Ritmejerytė et al. 2023).

An important next step in the field of pollination ecology will be to understand how pollen secondary metabolites are produced, stored, and controlled—in other words, where do they come from—and to determine what are the direct impacts of flower-colonizing microbes on plant reproductive success (i.e., impacts that are not mediated by pollinators). It will also be important to determine the heritability of defensive pollen traits and their intraindividual variability to better understand how these traits might evolve and respond to selection. It was not possible to test intraindividual variability in pollen alkaloid content in Chapter 3 because of the small amount of pollen produced by individual flowers of *L. argenteus*. Future studies could investigate intraindividual variability in pollen chemistry in plants with very high pollen production, such as those with large pollen-rewarding flowers pollinated by bats or bees. Another question that naturally stems from my findings in chapter 3 is: if pollinators do not

avoid pollen compounds with known toxic effects on insects, could they be adapted to tolerate such compounds?

6.2 Consequences of pollen defences

I was motivated to study the link between pollen defences and bee host-plant use because multiple researchers have pointed out the striking similarities in evolutionary patterns of food use between bees and herbivorous insects (Sipes and Tepedino 2005, Sedivy et al. 2008, 2013, Müller and Kuhlmann 2008). Since in herbivores such patterns are thought to be mainly driven by plant defences (Ehrlich and Raven 1964, Futuyma and Agrawal 2009), pollen defences could be a similar driver in the evolution of pollen-host use by bees. In Chapter 4 I aimed at testing whether bees are better adapted to tolerate host relative to non-host pollen defences in a group of bees with various host associations. I proposed that if bees experience limited mortality from host pollen defences but incur considerable mortality when fed non-host pollen, pollen defence should restrict host-plant use by bees. The assumption behind this reasoning is that switching between plant hosts should be constrained when such shifts incur strong fitness costs. I found that bees exhibit better tolerance of the defences of their pollen hosts relative to non-host defences, although some species seem to tolerate some non-host defences (which is also commonly observed in herbivores feeding on vegetative tissues; Cornell and Hawkins 2003).

Even with multiple years of data collection and laboratory manipulations, I was only able to investigate a single bee taxon and two pollen defences (pollen spines and quinolizidine alkaloids). This study alone therefore does not allow me to determine whether the role of pollen defences in the ability to exploit different potential hosts is restricted to Osmiini or if it represents a widespread phenomenon across bee taxa. Despite this, my findings complement

multiple studies across bee taxa that have observed similarities in host-use patterns between bees and other insect herbivores, or that found that bees are often unable to use non-host pollen (Sipes and Tepedino 2005, Praz et al. 2008, Sedivy et al. 2008, 2011, 2013, Müller and Kuhlmann 2008). Together, these findings suggest that pollen defence is an important and widespread driver of host-plant use by bees. Still, more data are needed to test the generality of my findings across bee taxa and pollen defences. One exciting new avenue for future research is to test the broad associations between pollen defences and bee host use at broad phylogenetic scales. This would require collecting considerable information on pollen chemical and mechanical defences, as well as bee host use and phylogeny. However, given my findings from this chapter, I believe such studies could significantly advance our understanding of the drivers of host associations in bees.

Considering their direct impact on bees'—and potentially other pollen consumers'—ability to exploit different plant hosts, pollen defences could have multiple ecological and evolutionary impacts on pollinators. The microbiomes of the bee gut and of the pollen provisions mediate bee nutrition, defence against pathogens, and detoxification (Engel et al. 2016, Raymann and Moran 2018, Steffan et al. 2019, Koch et al. 2022). Secondary metabolites toxic to insects often exhibit antimicrobial properties (Rivest and Forrest 2020). Pollen secondary chemistry could thus mediate the assembly of bee microbiomes. On the other hand, the detoxifying abilities of microbes could help bees exploit plant hosts with otherwise toxic pollen by reducing the concentration of harmful compounds. For example, Koch et al. (2022) found that both bees and their gut microbiome can contribute to the conversion of nectar secondary metabolites. Investigating how pollen secondary metabolites affect bee microbiomes and vice versa is a promising avenue for future research.

In a progressively more anthropogenically disturbed world, pollinator diets increasingly comprise pollen from non-native plants. Considerable effort has been devoted to understanding how invasive plant species affect pollinator health and abundance (Stout and Morales 2009, Stout and Tiedeken 2017). However, secondary chemistry is practically never considered as a potential factor in this equation. Yet, we are well aware that herbivorous insects (a group that includes bees) are usually adapted to tolerate the chemical defences of hosts with which they share an evolutionary history (Cornell and Hawkins 2003, Opitz and Müller 2009, Ali and Agrawal 2012). As I have shown in Chapter 4, bees are not able to tolerate the chemistry of all potential hosts. In anthropogenically disturbed habitats this should include invasive plant species with their unique secondary chemistry, to which pollinators have never before been exposed before. How these novel chemicals affect pollinator health remains an important unanswered question. A recent study by Tiedeken et al. (2016) found that secondary metabolites from the nectar of an invasive plant species can have detrimental effects on native pollinators, suggesting that more attention should be paid to plant reward chemistry in an anthropogenic context.

6.3 External drivers of the pollination mutualism

In Chapter 3, I provided evidence that factors external to the pairwise pollination mutualism could be important drivers in the evolution of this mutualism. Herbivores and microbes, in particular, appear to have potentially important ecological impacts on the pollination process. As I have pointed out at multiple occasions in this thesis, understanding how flower-colonizing microbes drive the evolution of the pollination mutualism, especially by mediating the evolution of floral traits, remains an important unanswered question.

My findings from Chapter 5 show that flower-colonizing microbes are unlikely to act as important drivers of flower evolution or to mediate pollinator-driven evolution. This is surprising considering that multiple studies have demonstrated that microbes can impact the interaction between plant and their pollinators (Vannette et al. 2012, Herrera et al. 2013, Schaeffer and Irwin 2014, Rering et al. 2018, Schaeffer et al. 2019, Yang et al. 2019, de Vega et al. 2022, Tsuji 2023), although few studies have shown a direct impact of microbes on plant reproductive success (but see Eisdcowitch et al. 1990). However, the impact of microbes on pollination in these ecological studies varies considerably, not only in intensity but also in direction (i.e., ranging from positive to negative), as a function of the species investigated and the ecological context. It seems likely that not only the ecological impacts of microbes on pollination, but also their evolutionary impacts might be highly species-specific, depending on how they impact plant–pollinator interactions. As with pollen defences, finding general patterns in the role of microbes in driving flower evolution might prove challenging.

This high variability in ecological interactions between plants and microbes might also prevent microbes from contributing significantly to flower evolution. Flowers are colonized by a diverse community of microbes, with the composition of this community varying considerably between flowers, even within the same individual. For example, flowers can vary from yeast-dominated to bacteria-dominated due to priority effect (Chappell et al. 2022), and both types of microbes have been found to have opposite effects on pollination (Vannette et al. 2012, Vannette and Fukami 2018). Combined with the drastic variation among flowers in microbial abundance (Russell and Ashman 2019, Chapter 5 of this thesis), this lack of consistency in flower microbial composition might not only result in variable microbe-driven selection among populations, but could also impede consistent selection within populations. It is not clear whether the use of

multiple microbe species weakened microbe-driven selection in Chapter 5 of this thesis (we should at least expect priority effects to play a negligible role in our system, but we did find high variability in microbial abundance). For example, yeasts and bacteria might have produced opposing selection on floral traits. Nevertheless, the complex and seemingly unpredictable nature of plant–pollinator–microbe interactions highlights the fact that, while experiments conducted in controlled conditions represent important proof-of-concept studies, they cannot take the place of experiments performed in the field.

I did not measure the evolution of pollen chemical defence in Chapter 5. The small amount of pollen produced by the line of fast-cycling *Brassica rapa* that we used would have made its chemical analysis at the individual level challenging (the plants that we used were smaller than in previous experimental evolution studies due to a change in the plant lines used by the vendor). However, my results suggest that the evolution of pollen secondary metabolites in response to flower-colonizing microbes is unlikely. We did not detect any floral evolution that would suggest that plants with lower microbial loads have a selective advantage. For example, the evolution of greater autonomous selfing in plants exposed to high microbial loads would have suggested a negative effect of microbes on pollination (as selfing generally evolves in response to low or unpredictable pollination service; Larson and Barrett 2000, Lloyd 2012), but we found the opposite trend. Hence, we can assume that no selection for stronger pollen defence against flower-colonizing microbes was taking place in my experimental evolution study. This conclusion contradicts my hypothesis from Chapters 2 and 3 that pollen-colonizing microbes could drive the evolution of pollen defences. However, as I discussed in Chapter 5 and in this section, multiple limitations in the experimental evolution study of this chapter might have limited my ability to detect pollinator- and microbe-driven evolution of floral traits. More studies

are needed that investigate the direct ecological impacts of flower-colonizing microbes on pollen germination and plant reproductive success.

6.4 Conclusion

Taken as a whole, my thesis suggests that factors external to the pollination mutualism—here herbivores and potentially microbes—have profound impacts on the ecology and evolution of this interaction. The idea that pollinators are not the only agents of selection on flowers is not new. Multiple studies have shown that non-pollinating animals can act as agents of selection on floral traits (Strauss and Whittall 2006). However, I am aware of few examples of interactors external to a mutualistic interaction impacting the structure and organization of this interaction in a way similar to what I demonstrate for bee host-plant use. Herbivores seem to be indirect drivers of pollen defences, as is evidenced by the correlated chemical defences between pollen and other plant tissues. Pollen defences resulting from plant–herbivore, and perhaps plant–microbe interactions, in turn seem to play a significant role in driving host use by bees, thereby affecting the structure of plant–pollinator interactions. Oppositely, I found minimal evidence of a role of microbes in mediating flower evolution, showing that some non-pollinating flower interactors may not play a major role in the evolution of pollination.

Extending our comprehension of ecological interactions from species pairs to whole communities is a monumental task, but one of great importance to our understanding of community ecology and to predict population dynamics. Understanding how pollen defences drive plant–pollinator interactions represents only a small contribution to this effort. Nevertheless, in doing so, my thesis contributes to answering long-standing questions in pollination ecology. For example, we still have very little idea why bees specialize in their pollen

use. By showing that pollen defences restrict the ability of some bee species to exploit different floral hosts, my thesis suggests that pollen defences might contribute to this still little-understood phenomenon. Moreover, resolving seemingly paradoxical phenomena in ecology often helps shift perspectives on how ecological processes operate. I hope that my work on the causes of pollen defences in Chapters 2 and 3 contributes to shifting our perspective away from pollination being a tight mutualism between plants and pollinators. As is often the case with new and existing fields of study, I think my research in this thesis raises at least as many questions as it answers, some of which I have presented in this Chapter. Understanding how plants, pollinators, microbes, and herbivores all interact together will require much work. My thesis represents one of the many steps towards achieving this goal.

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