INTEGRATION OF CHEMICAL INFORMATION: THE ROLE OF PLANT CHEMISTRY MEDIATING INTERACTIONS FROM THE INDIVIDUAL TO THE COMMUNITY LEVEL

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INTEGRATION OF CHEMICAL INFORMATION: THE ROLE OF PLANT CHEMISTRY MEDIATING INTERACTIONS FROM THE INDIVIDUAL TO THE COMMUNITY LEVEL

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Plants produce a large diversity of secondary metabolites that are increasingly understood to be important in the mediation of interactions with other organisms. The field of Chemical Ecology treats these interaction-mediating compounds as information that can be utilized by all organisms able to perceive and interpret it. For plants, this ubiquitous availability of chemical information can be compromising because the same info chemicals can, at the same time, function to attract mutualists, while they also have to function to minimize interactions with antagonists, such as competitors, herbivores, and pathogens. In this thesis, I first present a review of the evidence on such a plant chemistry-mediated ecological conflict; herbivory-induced changes in plant secondary metabolism and chemical resistance compromising interactions with pollinators. Then I use a case study from an Ericaceae shrub, *Becharia resinosa*, growing in endangered High Andean Forest habitats, to investigate interactions between florivores, nectar robbers, and pollinators mediated by a rare floral chemical trait, petal stickiness. Finally, I use a chemical information model system, the tall goldenrod, *Solidago altissima*, to study the integration of different types of environmental information used by the plant to affect the outcome of ecological interactions. Specifically, I study the perception of reduced red: far-red light ratios as a cue for competition and the perception of herbivore damage, to understand how plants integrate spectral and chemical environmental information. In addition, I show evidence that a plant's perception of

competitive neighborhood alters the perception and processing of chemical information encoded in volatile organic compounds from neighboring plants. All these studies have in common that they suggest that the outcome of ecological interactions mediated by chemical information strongly varies with the environmental context in which they are played out and how the information is utilized by the interacting players.

BIOGRAPHICAL SKETCH

Alexander Chautá Mellizo was born in Sesquilé, Colombia. After finishing high school, he entered Universidad Nacional de Colombia in Bogotá, Colombia to study Biology. He received a bachelor's degree in biology in April 2011. His thesis was focused on the effects that different pollinators had on the quality of fruits of cape gooseberry, *Physalis peruvianum*. This work was developed under the direction of Katja Poveda and Argenis Bonilla. In 2012, Alexander Chautá won the scholarship "Jovenes investigadores e innovadores" granted by Colciencias, which allow him to continue studying the pollination of cape gooseberry. He later attended the course on Tropical ecology and Conservation offered by the Organization of Tropical Studies in Costa Rica. In 2012, he started in the Master of Science program at Universidad Nacional de Colombia under the direction of Katja Poveda and Marisol Amaya, where he focused on studying the effects of herbivores on pollination in a hummingbird pollinated plant. In August 2015, he entered the Ecology and Evolutionary Biology program at Cornell University in Ithaca, NY, to study with André Kessler and continue his interest in the conflict between pollinators and herbivores. Specifically, he studies the outcome of ecological interactions mediated by chemical information in two different systems. One tropical plant in the high Andes and a common plant in the Northeast of USA.

To my parents for their infinite support

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Chapter 1

The ecological consequences of herbivore-induced plant responses on plant-pollinator interactions

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Abstract

Plant-induced responses to herbivory have long been found to function as plant direct and indirect defenses and to be major drivers of herbivore community and population dynamics. While induced defenses are generally understood as cost-saving strategies that allow plants to allocate valuable resources into defense expression, it recently became clear that, in particular, induced metabolic changes can come with significant ecological costs. In particular, interactions with mutualist pollinators can be significantly compromised by herbivore-induced changes in floral morphology and metabolism. We review recent findings on the evidence for ecological conflict between defending against herbivores and attracting pollinators while using similar modes of information transfer (e.g. spectral, olfactory, tactile). Specifically, we discuss plant traits and mechanisms through which plants mediate interactions between antagonists and mutualists and present functional hypotheses for how plants can overcome the resulting conflicts.

Introduction

Plants respond to environmental stresses with a multitude of changes to their primary and secondary metabolism (Karban and Baldwin 1997). The induced changes of secondary metabolism in response to biotic stressors, such as damage by herbivores and pathogens have received particular attention as those responses are increasingly found to boost the plants' ability to cope with antagonists (Karban and Baldwin 1997; Agrawal 1999). How induced changes in secondary metabolism can affect the outcome of an interaction between plants and their attackers can be multiple. (A) The changes can include the production of compounds that facilitate wound closure and reduce oxidative stress at the damage site by expressing increased amounts of antioxidant compounds and reducing primary metabolism (Kessler and Baldwin 2002a). This way metabolic changes simply help the plant to adjust to the new circumstance of being under attack and so assure structural integrity and reproductive success. (B) The metabolic changes can include the increased production of toxic, antidigestive and antinutritive compounds as well as a decreased primary metabolism that individually or in concert function as direct defenses by reducing the attackers’ performance or residence and so, secure increased fitness relative to plants that do not induce such change in secondary metabolite production (Karban and Baldwin 1997). (C) The metabolic change per se rather than the increased production of defensive compounds could function as a defense as the host search and adaption pattern changes constantly for the attacker in analogy to a moving target in military warfare (Adler and Karban 1994). (D) Finally, some of the changes in secondary metabolism can function as information mediating interactions with third-party organisms. Those could include third trophic level interactors, such as predators and parasitoids (indirect defenses) or neighboring plants (associational resistance, plant-to-plant communication), that indirectly reduce herbivore pressure (Kessler and Heil 2011).

All four hypotheses assume a significant cost of a constitutive production of defense-mediating compounds so that the inducibility of metabolic changes has traditionally been viewed as a cost-saving strategy (Karban and Baldwin 1997; Carmona and Fornoni 2013). Following this logic, plants only change their metabolism to an increased resistance mode when needed, in case of an actual attack. Such allocation costs of secondary metabolite production have been increasingly questioned in the light of similarly large costs associated with the maintenance of the machinery required to mount induced responses to environmental stress (Meldau et al. 2012). At the same time, however, the ecological and opportunity costs are frequently very high. For example, for a plant responding to one type of attacker can mean an inability to respond to another attacker. Similarly, some changes in plant secondary metabolism, such as increased volatile organic compound (VOC) emissions, can make the plant more visible to attackers and can so function as aggregation cues (Loughrin et al. 1995; Halitschke et al. 2008). One ecological cost category, specifically important to this paper is the potential negative effects that herbivory-induced responses can have on interactions with mutualistic organisms, such as natural enemies of herbivores, microbes, and pollinators, the latter being the focus of our discussion. Plant responses to damage by herbivores and pathogens involve a significant transcriptional and metabolic reconfiguration of the plant that is regulated by a complex interactive network of endogenous plant signals (Kessler and Baldwin 2002a). These changes have two major ecological consequences: First, they can change the suitability of the plant as a food source or habitat. Second, they can change the information landscape that allows detectability and identification of appropriate hosts to interact with (Kessler 2015). In both cases, the induced changes can alter the status of interactions and thus the community and community dynamics of both antagonists and mutualists associated with the plant. A particularly interesting example of this type of potentially conflicting consequences is the effect of herbivore-induced changes in plants on pollinators (Kessler and Halitschke 2009; Johnson et al. 2015; Schiestl 2015; Lucas-Barbosa 2016; Jacobsen and Raguso 2018; Rusman et al. 2019a). That herbivory can affect pollinator attraction has been well-established pollinators (Strauss et al. 1996, 2002; Kessler and Halitschke 2009; Schiestl 2015; Lucas-Barbosa 2016; Moreira et al. 2019) but it remains still little studied which floral traits link herbivory to pollinator attraction and what consequences this type of phenotypic plasticity has for the evolution of floral traits. Damage by herbivores can directly affect pollinators by fully or partially destroying floral tissues and rendering floral structure and rewards unsuitable (Strauss et al. 1996). Such cases can thus be considered as direct fitness effects of herbivory that are not truly mediated by pollinators. More interestingly, however, by consuming plant tissues (pollen) and products (nectar, oils, volatile compounds) or by simply touching plant tissue when approaching a rewarding flower, pollinators are exposed to similar plant defensive metabolites as herbivores. Moreover, responses to environmental stresses, such as herbivory and the development of the entire plant and its reproductive structures are regulated by phytohormonal and biosynthetic pathway cross-talk using the same basic multifunctional phytohormones, such as jasmonic acid, salicylic acid, ethylene, abscisic acid, and indole acetic acid (Rusman et al. 2019a). As a consequence, the plants' phytohormonal and metabolic changes in response to herbivory affect growth, phenology, and volatile chemistry of flowers, which alters the information available to pollinators to find and remember flowers. This means that if flower morphology, the chemistry of floral rewards, and signaling change, pollinator behavior can be affected, and plants can suffer an indirect cost from herbivory by mounting induced responses to their attackers. Thus costs of herbivory can be ‘larger than the sum of their holes’, not only because of the loss of photosynthetic tissue and the commonly observed down-regulation of primary metabolism (Zangerl et al. 2002) but also because of the ecological costs associated with herbivory-induced changes in flower morphology, phenology and secondary metabolite production (Kessler and Halitschke 2009). Although a majority yet limited number of studies to date seem to report negative effects of herbivory on pollinator attraction (Lucas-Barbosa 2016; Jacobsen and Raguso 2018; Moreira et al. 2019), there is also clear evidence that the ecological outcome of induced responses depends on plant species, type of herbivore damage and type of tissue that is damaged (Poveda et al. 2003; Gegear et al. 2007; Moreira et al. 2019; Rusman et al. 2019b, a) Why those differences in ecological consequences of herbivory occur, is still largely unclear. However, the ecological context, such as the availability of alternative flower hosts in a plant community (Gegear et al. 2007) or the relative abundance of damaged vs. undamaged plants within a plant population, are likely to affect the outcome of the interactions between plants, herbivores and pollinators mediated by plant induced responses to herbivory (Kessler et al. 2011). Here, we review herbivore-induced changes in plant traits important to pollinators and their ecological effects to assess their relative importance in driving plant–herbivore–pollinator dynamics and so the evolution of plant reproductive traits. We specifically examine four categories of herbivore-induce changes: plant growth (phenology and morphology), reward production, defensive secondary metabolite production and floral signaling.

Herbivory-induced changes in flower phenology and morphology

Herbivory can have fundamental effects on plant growth. On one hand, this results from the mere removal of photosynthetically active (aboveground) or nutrient-absorbing tissues (belowground) by herbivores. On the other hand, tissue damage triggers complex phytohormonal interactions that result in a reallocation of resources, frequently away from primary metabolism and towards secondary metabolism (Kessler and Baldwin 2002b). In consequence, the remaining tissues on damaged leaves frequently contribute less to the overall growth and reproduction budget of the plant. The herbivory-induced differential reallocation of resources into different plant life functions out of a finite resource pool is viewed as optimization of plant metabolism to maximize net fitness (e.g. optimal defense theory) (Stamp 2003). However, this is assuming that the major costs associated with those changes are physiological or opportunity costs. They do not necessarily consider the potentially negative effects of those changes on ecological interactions of the plant with other organisms (e.g. ecological costs), such as pollinators. However, the differential expression of phytohormones can have fundamental effects on plant growth (e.g. floral display) and phenology which, in turn, can affect the interactions with pollinators (Rusman et al. 2019a). But how big can these effects be?.

Many recent studies that manipulated pollinators as the agents of natural selection found display size (Leimu et al. 2006; Parachnowitsch and Kessler 2010; Bartkowska and Johnston 2012), flower color (Caruso et al. 2010; Sletvold et al. 2016), and floral phenology (Parachnowitsch et al. 2012b; Sletvold et al. 2012) under strong selection, suggesting that phenotypic plasticity in those traits can affect pollinator behavior and, in consequence, the fitness outcome for a plant. Leaf herbivory has indeed been found to induce reductions in the number of flowers as well as corolla size, which is usually associated with a reduced seed set per flower (Strauss et al. 1996; Mothershead and Marquis 2000). This fitness reduction can result from a limitation in resource allocation for reproductive tissues, such as reduced ovary sizes, or reduced pollinator attraction due to alteration in reward, spectral and olfactory cues. The changes in floral display size are commonly associated with a reduced pollinator attraction and, in consequence, a significant reduction in seed set. For example, studies in *Oenothera macrocarpa* established such a link and found that the fitness-reducing effects are the result of pollen limitation, rather than plant resource allocation (Mothershead and Marquis 2000). Moreover, different pollinator species may respond differently to specific herbivory-induced changes in flower morphology, so that herbivory can alter pollinator community composition and thus not only affect pollinator recruitment in general but also pollinator fidelity (Strauss et al. 1996).

A similar shift in pollinator availability and community composition can be expected when flowering phenology changes in response to herbivory. Genotypic differences in flower phenology are often found to have large effects on pollinator availability and flower herbivory with delayed flowering reducing both herbivory and pollination ((Mothershead and Marquis 2000; Parachnowitsch et al. 2012a, but see Toivonen et al. 2019). Similarly, phenotypically plastic responses to leaf herbivory can affect flower phenology in both directions and cause earlier flowering as well as delayed flowering with quite different ecological consequences (Freeman et al. 2003). Delayed flowering can move reproduction so close to the end of the growing season that pollinators may be limited or fertilization too late to allow fruits sufficient time to develop. Flowering earlier in response to herbivory can on one hand move the flowering peak away from peak pollinator availability and so cause pollen limitation. On the other hand, accelerated flowering can mean a more synchronized mass blooming in species with multiple flowers and so a relatively larger floral display that is more attractive to pollinators (Paige and Whitham 1987). Such accelerated mass blooming has been hypothesized as a mechanism for plants to overcome the negative effects associated with leaf herbivory (Schiestl et al. 2014). A recent study found that resource-limited bumblebees, *Bombus terrestris*, cut leaves to induce accelerated flower production in several plant species (Pashalidou et al. 2020). This remark-able behavior does not only increase the availability of pollen rewards for the bumblebees but potentially also allows plants to time their flowering with the peak activity of the pollinators. Thus, in this case, induced responses to leaf tissue damage could directly benefit the plant by facilitating its interaction with pollinators rather than generating ecological costs. Leaf tissue damage has also been shown to affect herkogamy of the reproductive structures in hermaphroditic flowers. For example, pistil length in flowers of plants of the wild tomato species *Solanum peruvianum* that are attacked by leaf herbivores is shorter than in flowers on undamaged plants. As the anther cones do not change in length, herbivory changes flower morphology from approach herkogamy (pin) to anthers and stigma on the same level. As this morphological change can increase selfing rates, herbivore-induced changes in herkogamy have also been hypothesized as a plant mechanism that allows mediating negative effects of herbivore attacks on pollination (Kessler and Halitschke 2009). Here, as a kind of herbivore-induced reproductive assurance, it is hypothesized that an induced change in flower morphology helps compensate for reduced pollinator attraction by increasing selfing rates. However, similar changes in herkogamy in other systems can also mean that pollen success will be limited. In the Andean Rubiaceae, *Palicaurea angustifolia*, herbivory-induced changes in herkogamy are thought to limit pollination and thus negatively affect seed set (Chautá et al. 2017) (Fig. 1.1).

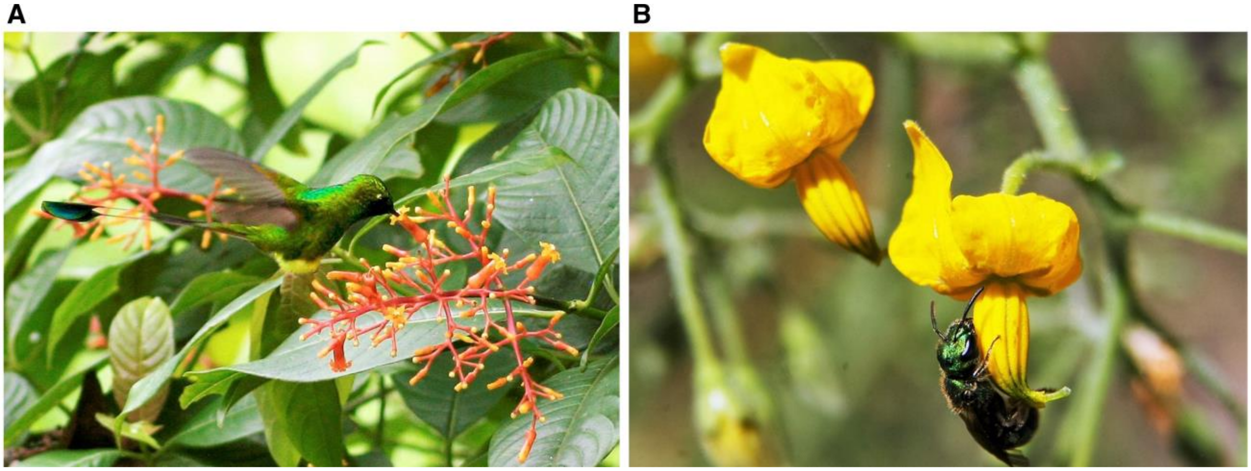

Figure 1.1. Plant induced responses to herbivory affecting pollinator visitation. (A) The hummingbird *Ocreatus underwoodii* feeding on the ornithophilic species *Palicourea* sp. (Rubiaceae). Herbivory reduces hummingbirds visitation due to an induced reduction in the amount of nectar (Chautá et al. 2017). (B) A *Halictidae* bee visiting a wild tomato, *Solanum peruvianum* (Solanaceae) flower. Leaf herbivory induces changes in floral and vegetative volatile organic compound emission. This change in chemical information causes pollinators to avoid flowers on herbivore-damaged plants, causing reduced seed set due to pollen limitation (Kessler et al. 2011).

Floral coloration is another trait that can affect pollinator attraction. For example, florivores of *Raphanus sativus* prefer and perform better on plants with yellow or white flowers compared with pink and bronze flowers, due to associated differences in the amount of defense-mediating indole glucosinolates (Irwin et al. 2003). Moreover, anthocyanins can play an important role in defending against florivores (Johnson et al. 2008). However, to our knowledge, no functional, herbivore-induced changes in flower coloration have been observed so far but could be expected given the commonly dramatic changes in plant metabolism in response to herbivory.

Leaf herbivore-induced alterations in floral morphology and phenology can affect both the attractiveness of flowers to pollinators and the pollination success when pollinators interact with flowers (Kessler and Halitschke 2009). In how far this type of phenotypic plasticity affects plant fitness and the evolution of plant traits likely depends on the reproductive strategy of the plant and the behavioral flexibility (persistence) of the associated pollinator community but is still little understood (Campbell 2015; Schiestl 2015; Jacobsen and Raguso 2018). Such an understanding centers around the question of whether or not these herbivore-induced changes in flower trait expression with consequences for pollinator attraction can be adaptive or are merely ecological costs associated with a plant's adjustments of physiology and metabolism to the attack by herbivores. And, this question can certainly be expanded to actual pollinator rewards and the chemical traits of flowers, the ecological functions of which are thought to interact with those of morphological traits (Parachnowitsch et al. 2012b; Ramos and Schiestl 2019).

Variation in reward quantity

Although plants have evolved a wide variety of nutritive and non-nutritive rewards (Simpson and Neff 1981) to attract and retain pollinators, nectar and pollen are by far the most widespread rewards offered to pollinators of Angiosperms (Irwin et al. 2004). Despite this generalizable importance and the functional link to pollinator attraction, the heritability of and natural selection on nectar, pollen, and their associated characteristics such as sugar, and amino acid concentration, or secondary metabolite production is poorly understood (Parachnowitsch et al. 2019). In part, this knowledge gap derives from difficulties to identify relevant nectar and pollen traits and how to measure them (Mitchell 2004). However, probably more important is the fact that nectar/pollen production and composition can vary strongly with changing environmental circumstances. For example, abiotic factors, such as light (Pleasants 1983; Devlin 1988), temperature (Jakobsen 1994; Petanidou and Smets 1996), humidity (Bertsch 1983), water availability (Carroll et al. 2001; Waser and Price 2016) and CO2 concentration (Dag et al.; Rusterholz and Erhardt 1998) can all affect nectar productions, suggesting that microclimate through changes and endogenous signaling and plant metabolism has a large impact on phenotypic plasticity of rewards. In addition, biotic factors, such as soil microbes (Baude et al. 2011; Becklin et al. 2011) affecting nutrient availability and metabolite production as well as microbes (Vannette et al. 2013; Vannette and Fukami 2018) directly living in and on pollinator rewards can alter the reward availability and composition. Most interesting for this review, however, is the evidence for herbivory affecting reward availability and quality.

Much like morphological traits, pollen and nectar availability can be affected by herbivore damage. For example, defoliation in the Peruvian lilly, *Alstroemeria aurea*, reduced the size of the flowers, lowered nectar production, and made pollen grains smaller with negative effects on pollinator attractiveness (Aizen and Raffaele 1996) and pollen viability (Aizen and Raffaele 1998). Similarly, reduced style length and an associated reduced pollen deposition, as well as a reduced nectar production that attracted fewer hummingbird pollinators, were identified as major mechanisms for reduced seed set in *P. angustifolia* (Chautá et al. 2017). Thus, reduced reward production and altered floral morphology seem to be commonly associated and may function in concert with reduce pollinator attraction. However, in the wild mustard, Sinapis arvensis, nectar production was not affected by leaf or root herbivores (Poveda et al. 2005), while pollinator attraction and resulting seed set was affected negatively by leaf herbivory and positively by root herbivory (Poveda et al. 2003). Moreover, reduced nectar production after herbivory can also increase outcrossing rates because pollinators would have to visit more flowers or travel farther to obtain enough nectar (Cushman and Beattie 1991; Maloof 2001). These examples do not only demonstrate a dependency of herbivore-induced changes in floral traits from differential endogenous signaling associated with above and below-ground herbivory (Kaplan et al. 2008b, a). They also suggest the potential existence of additional floral traits and their integrated perception by the foraging pollinators.

Herbivore–pollinator cross-resistance

One of the trait complexes of increasingly recognized importance, especially considering the plant-mediated interactions between herbivores and pollinators is floral chemistry (Adler 2000; Raguso 2008; Kessler and Halitschke 2009; Theis et al. 2014; Schiestl 2015; Jacobsen and Raguso 2018). Pleiotropy has been hypothesized to tightly link floral to leaf chemistry. Thus increased natural selection by herbivores on leaf defense chemistry can be predicted to also affect interactions with pollinators (Kessler and Halitschke 2009; Schiestl and Johnson 2013; Johnson et al. 2015; Campbell 2015). Macroevolutionary, this would mean a strong interaction between mating system and defense strategy in plants. Indeed, recent phylogenetic comparisons of the genus Nicotiana revealed that plants with greater dependence on pollinators produce relatively lower amounts of defensive metabolites than plants that do not rely on animal pollinators (Adler et al. 2006). A similar study across a larger set of Solanaceae taxa found that a phylogenetic, unidirectional shift from self-incompatibility to self-compatibility was associated with the evolution of inducible resistance strategies and decreased constitutive resistance. Moreover, the loss of self-incompatibility was also associated with a higher specificity of plant responses to different types of leaf tissue damage (Campbell and Kessler 2013). While both studies support the hypothesis that pleiotropy in secondary metabolite production among vegetative and reproductive tissues link the evolution of defensive strategies to that of plant mating systems, the later study also bears a clear indication that, at least on a macroevolutionary level, inducibility of resistance to herbivory can come with the ecological cost of pollinator limitation.

Accordingly and on an individual plant level, while floral secondary metabolism frequently differs to a limited extent from leaf secondary metabolism (Kessler and Halitschke 2009), increased production in response to herbivory and an associated increased resistance to both herbivores and pollinators can be observed. For example, in studies with the tobacco species, *Nicotiana tabacum* (Adler et al. 2006) and *N. quadrivalvis* (Halpern et al. 2010), herbivory induces increased alkaloid production in the nectar reward, suggesting similar underlying regulatory induction processes as well as a strong link between herbivory and pollination. Other studies found no differences in the reward chemistry while effects on pollinators remained (Kessler et al. 2011; Bruinsma et al. 2014, Fig. 1.1B). At this point, the studies demonstrating changes in nectar chemistry in response to herbivory are very limited and not sufficient to draw generalizations. However, there is a fast-growing body of literature demonstrating that secondary metabolites in nectar have multiple functions and affect pollinator behavior and learning (Adler 2000; Stevenson et al. 2017). It is not yet clear how far secondary metabolite concentrations in reward tissues (e.g. nectar and pollen) change in response to herbivory and in how far those changes are ecologically relevant, specifically in relation to other floral traits, such as morphology, reward amounts, and signaling. For example, reduced nectar flow and increased nectar secondary metabolite production could have a similar effect on overall pollinator attraction, but the fitness consequences for the plant could vary quite widely. In wild tobacco, *Nicotiana attenuata*, a silencing of nicotine production in nectar led to increased pollinator residence on flowers and nectar removal but resulted in lower outcrossing rates for the plant (Kessler and Baldwin 2007). Moreover, the effects of the insect-repellent nicotine interacted with the hawkmoth-attractive benzaldehyde in nectar to optimize the attraction of pollinators and maximize outcrossing and seed set (Kessler et al. 2008). This suggests that herbivore-induced changes in reward secondary metabolites, in interaction with those of other floral traits, can trigger complex responses by the pollinator community and, in consequence, have a variety of outcomes for the plant. The predictability of these outcomes for a given community interacting with a plant would then depend on the relative importance of each inducible trait to an interacting organism (relative strength) and whether or not the induced changes function in the same direction (directionality). Natural selection can then be expected to affect the inducibility of those traits or trait combinations that most affect plant fitness-associated pollinator behavior. While not addressing inducibility, a recent study using a common garden experiment with *Brassica rapa* and manipulating pollinator availability and herbivore presence as agents of natural selection illustrates the complexity of such triangular interactions for the evolution of plant traits (Ramos and Schiestl 2019). Natural selection by bee pollinators alone resulted in an increased mean floral attractiveness, while the additional presence of herbivores selected for reduced herkogamy and the associated increased self-compatibility and autonomous selfing (Ramos and Schiestl 2019). Inducibility of defensive metabolites and floral morphology in leaves and floral rewards can thus also be predicted to be under natural selection by pollinators and herbivores alike. This notion is even further supported by the fact that herbivore-induced changes to pollinator interactions can have far-reaching ecological effects for the stability and resilience of interactions and communities (Glaum and Kessler 2017).

Mixed chemical messaging

Probably the most consequential but least studied effects of plant responses on pollinators and herbivores come with the alteration of the information space that is shared by all the interactors (e.g. VOCs, reward chemistry correlated with leaf chemistry). Plant-induced responses to herbivory usually involve a change in VOC bouquets emitted from plants (Dicke and Baldwin 2010; Kessler and Heil 2011; Rusman et al. 2019a; Bouwmeester et al. 2019). Herbivore-induced VOC emissions from vegetative and floral tissues are a reflection of the plant's metabolic status, including ongoing attacks by herbivores and pathogens, so that three major hypotheses for why pollinators would avoid flowers on damaged plants based on VOC emission alone can be proposed. First, pollinators can use VOC emissions to get information about the floral reward quality or, as in cases when pollinators are also the major herbivores, they can get information about the overall status of the plant as potential food (Adler et al. 2006; Kessler et al. 2008). Second, herbivore-induced VOC emission is known to attract predators that can attack herbivores to the benefit of the plant but may also attack or repel pollinators (Kessler and Heil 2011). Third, VOCs have been recently found to play fundamental roles in attracting pollinators (Raguso 2008) and maintaining their floral constancy (Wright and Schiestl 2009). The latter is particularly relevant to our discussion here. When pollinators associate a particular floral scent with a reward, relatively minimal changes to the VOC bouquet can disrupt the pollinator's search pattern and make a flower less attractive, with potentially negative consequences for pollination (Wright and Schiestl 2009). Although fundamentally different, the three mechanisms all explain the negative effect of herbivory on pollinator behavior. As several cases of herbivore-induced pollinator limitation and negative consequences to plant fitness have been reported (Lucas-Barbosa 2016), the question arises of how plants can overcome this apparent conflict of shared signaling. One way certainly is that plants can escape herbivory by either evolving very effective resistance traits or by occupying new, herbivore-free habitats. Similarly, plants can evolve to become relatively independent of pollinators by, for example, wind pollinating, selfing or reproducing primarily vegetatively. While these options can come with other problems for the plant (e.g. escaping herbivores may also mean escaping most pollinators, inbreeding depression, limited adaptability), most other plant species probably fall into two categories with clear predictions on inducible chemical signaling (Fig. 1.2). On one hand, there is the possibility that induced reduction in pollination as a function of herbivory could be adaptive if reproduction under stressful herbivore attack comes with opportunity costs on future reproduction (Kessler and Halitschke 2009). In such cases, floral signaling chemistry should be under selection to strongly vary with overall plant metabolism. On the other hand, if such opportunity costs are small or can be otherwise overcome (Knauer and Schiestl 2017; Ramos and Schiestl 2019), plants should be under strong selection to differentiate floral signaling metabolism from overall plant metabolism both, to clearly separate constitutive floral from vegetative VOC emissions and to limit inducibility of floral signaling when leaf tissue is attacked by herbivores (Fig. 1.2B). Thereby, both, the selectivity of VOC bouquet generation by the plant (Wee et al. 2018) and the selectivity in perception by the interacting organisms (Wilson et al. 2018) can mediate increased information noise due to induced metabolic responses of the plant. This further suggests that plants that have the physiological capability to separate floral from vegetative metabolism can evolve higher floral specificity as the strength of selection by herbivory increases. This is because metabolic differentiation partially removes herbivory as one of the major agents of diffuse natural selection on floral metabolism (e.g. removed flower-leave metabolic pleiotropy) and leaves pollinators as the major agents of selection. Flower traits are relatively more open to a runaway co-evolutionary process with pollinators. The opposite is expected to happen to plants without the capability to differentiate floral from vegetative metabolism, whit a higher metabolic specificity at lower levels of selection by herbivores (Fig. 1.2C).

Alternatively, the inducibility of resistance and the associated VOC signaling can function as a strategy of saving potentially high costs of a constitutive expression of the resistance-mediating trait as predicted by optimal defense theory (Karban and Baldwin 1997; Stamp 2003). This can apply primarily to plant species in which leaf and floral chemistry are closely linked and both genotypic, as well as inducible variation in signaling, affects pollinator behavior. The ecological costs of induced metabolic changes and reduced pollinator attraction would only be paid by the plant when herbivory is present. According to optimal defense theory, this should only be beneficial in habitats with relatively low or unpredictable herbivory (Karban and Baldwin 1997).

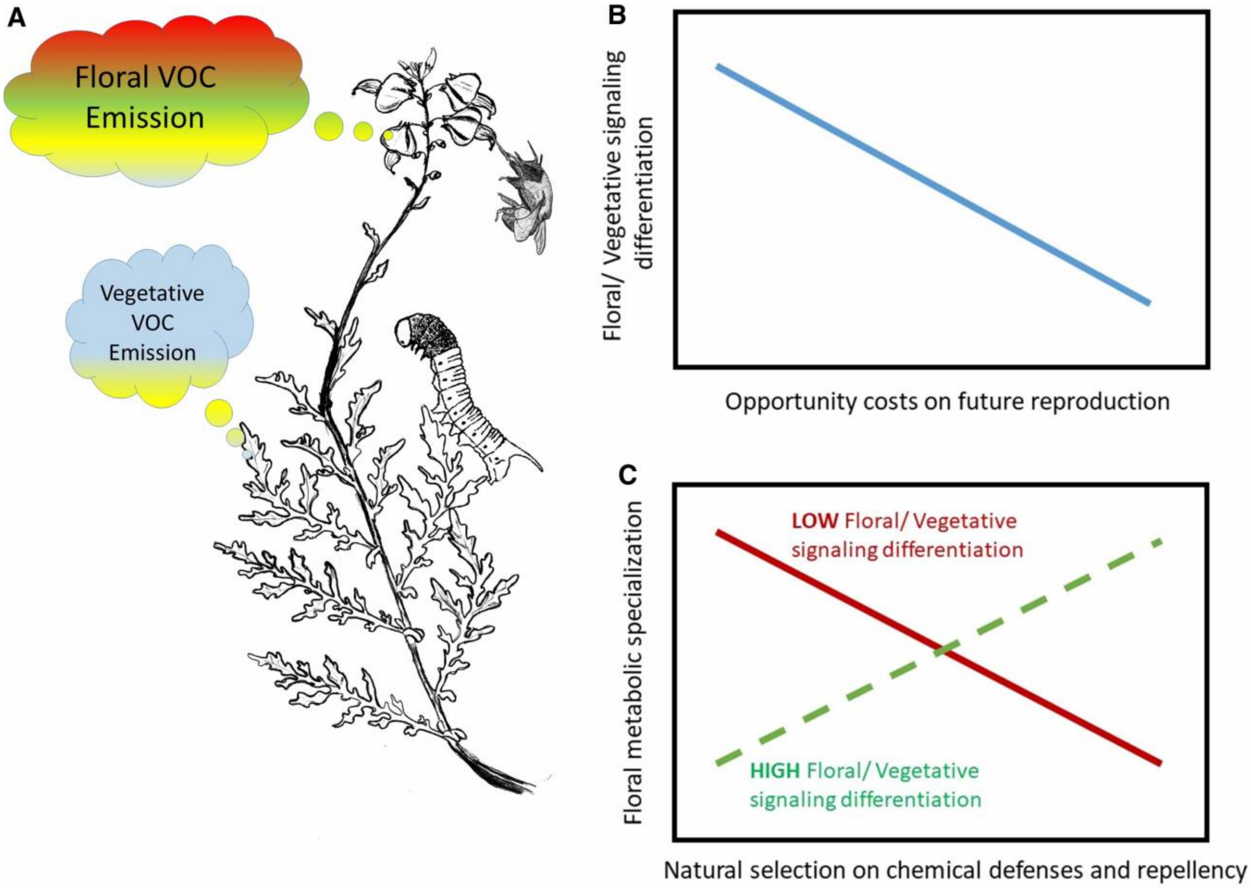

Figure 1.2. Conflict between repelling herbivores and attracting pollinators with a similar bouquet of chemical information. (A) Herbivory can induce changes in vegetative and floral volatile organic compound (VOC) emissions, which can lead to conflicting information noise and

thus the breakdown of the beneficial plant–pollinator interaction. Colored bubbles represent VOC bouquets emitted from flowers (top) and leaves (bottom). Similar colors represent similar compounds. Metabolic differentiation between floral and vegetative VOC emissions is a functional way for plants to avoid signaling conflict. The larger the difference between VOC bouquets of flowers and leaves the lower the conflict. (B) Floral: vegetative signaling differentiation is a function of the opportunity costs on future reproduction, with lower costs allowing relatively stronger pollinator-mediated selection on floral signaling. (C) Floral metabolic specialization (e.g. reduction of noise for pollinators or more specialized plant-pollinator interactions) is a function of the strength of natural selection on chemical defenses. Thereby predictions for selection gradients are opposite for plant species with high versus low floral: vegetative signaling differentiation.

Conclusion

Studies begin to provide clear empirical evidence that both pollinators and herbivores are major agents of natural selection on floral traits (Ramos and Schiestl 2019). The observed effects that herbivory-induced changes have on pollinator behavior and plant reproductive success underline this tight relationship but also illustrate the special role of plant secondary metabolism in this tripartite ecological and evolutionary interplay. So far, these herbivore-induced changes are largely thought of as ecological costs because they often compromise interactions with pollinators. However, a more targeted functional analysis of inducibility as a plant characteristic may reveal broader, adaptive roles of inducible traits in the plant’s interactions with pollinators. For example, developmentally controlled or induced-floral VOC emissions may have fitness-enhancing and context-dependent roles in mediating interactions between plants by synchronizing flowering or coordinating defense responses (Caruso and

Parachnowitsch 2016) as has been recently found for information exchange via herbivore-induced leaf VOCs (Kalske et al. 2019). Moreover, inducibility has been hypothesized to change the community and population dynamics of herbivores interacting with plants. In multi-species interactions involving antagonists and mutualists or multiple trophic levels, this can result in complex outcomes, including increased stability and resilience of the populations involved in the interaction (Glaum and Kessler 2017). Finally, pollinators can use damage inducibility of floral trait expression to their own and potentially the plants' advantage by synchronizing pollinator activity with flower resource availability (Pashalidou et al. 2020). All these newer studies begin to draw a picture of the immense importance of herbivore-induced changes of flora morphology and metabolism for the outcome of interactions with pollinators. Going forward innovative experimental approaches on studying micro and macro evolutionary patterns as well as ecological dynamics and underlying molecular and metabolic mechanisms begin to bring this new picture in focus but also establish plant-herbivore–pollinator interactions as a general model for conflicting selection on traits that mediate interactions with antagonists and mutualists alike.

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Chapter 2

A sticky story. defensive functions and ecological conflicts of adhesive chemicals in flowers of *Bejaria resinosa* (Ericaceae)

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Abstract

Stickiness of vegetative tissues has evolved multiple times in different plant families but is rare and understudied on flowers. While stickiness is thought to function primarily as a defense against herbivores, it can significantly compromise interactions with mutualist organisms, such as pollinators, especially when expressed in reproductive tissues. *Bejaria resinosa* (Ericaceae) is a High-Andean plant species that is dimorphic for stickiness on its flower petals, allowing for functional analysis of floral stickiness. Here, we hypothesize that stickiness on *B. resinosa* flowers functions as a defense against arthropod florivores, assess ecological consequences, and discuss potential trade-offs. In surveys and manipulative experiments, we assessed florivory and resulting fitness effects on plants with sticky and non-sticky flowers across three different populations of *B. resinosa* in the high Andes of Colombia. Additionally, we analyzed the volatile and non-volatile components in both morphs to understand the chemical information context within which stickiness is expressed. Our results causally link stickiness to reduced florivory, but also indicate high population context-dependency of the effect. In consequence, the fruit set was strongly affected by floral stickiness but also varied with the population. We discuss how this context-dependency likely arises from differential proportional availability of primary pollinators (hummingbirds, insects). Lab experiments with generalist herbivores found that sticky plants can be more palatable when herbivores are allowed to avoid their stickiness, suggesting a trade-off between stickiness and toxic metabolite production as two direct defense traits. Finally, stickiness in *B. resinosa* is linked to significant differences in non-volatile chemistry but not associated with differential volatile organic compound emissions. This study demonstrates a strong anti-florivore deterrent function of floral stickiness but points to potentially high ecological costs that may vary with the relative abundances of the major mutualists interacting with the plant.

Keywords: ecological conflicts, hummingbird pollination, indirect defenses, plant defense, pollination, protocarnivory.

Introduction

Plants have evolved multiple strategies to cope with herbivores. These strategies range from morphological defenses (e.g. thorns and trichomes) over tolerance to a complex set of secondary metabolites that can mediate direct and indirect resistance and repellency. Direct defensive secondary metabolites function through different mechanisms and include antinutritive, antidigestive, repellent, or physical-chemical hybrid traits. Physical-chemical hybrid defenses include surface waxes (Gorb and Gorb 2017), latex (Agrawal et al. 2008; Agrawal and Konno 2009), resin (Langenheim 1990), and sticky compounds exuded from trichomes (Leckie et al. 2016; Luu et al. 2017; Ben-Mahmoud et al. 2018) or epidermis cells that prevent insects to access the plant tissues surface or compromise mobility (Fig. 2.1). The latter, sticky compounds, are particularly interesting for their interesting ecological and evolutionary implications yet are poorly studied. The stickiness of plant vegetative surfaces is not rare and can be found in a diversity of species across different plant families (LoPresti et al. 2015). Sticky compounds are most commonly exuded by glandular trichomes, but also by specialized glands that produce compounds like acyl sugars, terpenoids, phenolics, and ketones (Kennedy 2003; Weinhold and Baldwin 2011; Krimmel and Wheeler 2015). Several functional hypotheses on the production of leaf surface adhesive compounds have been proposed.

First, sticky compounds on leaf surfaces can function as a direct defense and repel or kill antagonists, such as herbivores. Sticky plants can trap herbivores and kill them by starvation (Simmons et al. 2004) or the adhesive can include toxic substances and so affect antagonists that get stuck on or ingest those compounds. For example, sesquiterpenes found in glandular trichomes of wild tomato species have been found to increase mortality of Colorado potato beetle (Carter et al. 1989). Also, the presence of glandular trichomes on *Datura wrightii* was found to reduce the performance of *Manduca sexta* by increasing the time to pupation and reducing consumption rates (Van Dam and Hare 1998).

Figure 2.1. Insects in sticky flowers of *Bejaria resinosa.* **a.** Multiple flies, **b.** Bee, **c.** wasp, **d.** spider.

Second, arthropods trapped on sticky plant surfaces may attract predators that then further reduce herbivore pressure on the plant (LoPresti et al. 2015). In such cases, compounds mediating the adherence of prey organisms and so facilitating the residence of predators can be viewed as indirect defense traits (Kessler and Heil 2011). In some cases, the number of predators on sticky plants was found to proportionately increase with the number of insects stuck to the plant (Karban et al. 2019). The attraction of predators can reduce herbivore abundances and thus herbivory beyond the direct defensive effects of sticky compounds with potential additive or synergistic effects on plant fitness (Krimmel and Pearse 2013; LoPresti et al. 2015). A similar indirect defensive function of sticky compounds can be assigned in cases where volatile breakdown products of sticky compounds ingested by herbivores can function as prey-finding cues. For example, *O*-acyl sugars produced by trichomes of the wild tobacco *Nicotiana attenuata* are converted into a volatile branched-chain aliphatic acid in the guts of *Manduca sexta* caterpillars*.* An omnivorous ant, *Pogonomyrmex rugosus,* has been found to use this volatile cue to locate its caterpillar prey (Weinhold and Baldwin 2011).

Third, stickiness can help plants with acquiring additional nutrients. Soil nitrogen availability is limited in many natural soil types. However, in particularly limited habitats, plants have evolved ways of acquiring alternative resources of nitrogen, such as from captured animals. This strategy is notorious for carnivorous plants, but it is also found in some sticky plants without specified prey-capture organs. For example, *Geranium viscosissimum*, *Potentilla arguta* and *Stylidium* sp. can partially digest the proteins of insects trapped in their glandular trichomes (Spomer 1999; Darnowski et al. 2006). While these plants are capturing and digesting insects, they are not considered carnivorous but rather protocarnivorous, because the primary role of stickiness is assumed not to be capturing prey for supplemental nutrition (Givnish et al. 1984). To our knowledge, a thorough cost-benefit assessment of the different ecological functions of stickiness in plants has not yet been conducted.

Despite the seemingly obvious benefits of sticky compounds on leaf surfaces, there are also some associated ecological costs. For example, sticky surfaces can trap dust that could potentially affect light availability for photosynthesis (Lopresti and Karban 2016). In addition, mutualists could be affected by stickiness with potential direct negative effects to plant fitness (Lopresti and Karban 2016). Such ecological costs are probably the reason for the fact that while stickiness is a common trait of plant vegetative tissues, it is much less common in flowers (e.g. *Erica tetralix, Ceratotheca triloba*, *Ibicella* *lutea* and *Salvia glutinosa*). Ultimately, flowers are tissues that have evolved to facilitate interactions with mutualists. On the other hand, out of all the plant tissues, the herbivore damage to the flowers can be the most deleterious. Herbivores can reduce plant fitness significantly by imposing even minute amounts of damage to reproductive parts (e.g. anthers, style, ovaries) or when damaging corolla or colored brackets through changing the information transfer between plant and pollinators (Krupnick and Weis 1999; McCall 2008). This is hypothesized as a reason for why floral tissues are usually found to be more strongly constitutively defended with secondary metabolites (e.g. phenolic compounds, glycosides, soluble myrosinases) (Bandeili & Müller, 2010; Tsuji & Sota, 2010; Lai *et al.*, 2015, Kessler & Halitschke., 2009 but see Godschalx *et al.*, 2016) and deterrent volatiles (Kessler et al. 2013; Li et al. 2019) than leaf tissues.

However, the rarity of stickiness in flowers is likely due to the more direct negative effects on potential pollinators. Stickiness can trap insect pollinators reducing plant pollination and fitness. More importantly to this study, the stickiness can also affect interactions of the plant with other mutualists, by, for example, entrapping small parasitoids (Kennedy 2003) or reducing predator mobility (Kennedy 2003), thus compromising indirect defenses. Because of this more directly played out conflict between repelling antagonists and attracting mutualists, interactions of plants that feature sticky floral parts with their interacting community provide ideal models to understand the ecological dynamics of conflicting interactions mediated by plant chemistry. Nevertheless, floral stickiness is poorly studied both functionally (including basic anti-florivore defense function) and mechanistically.

Bejaria resinosa (Ericaceae) is a species that produces a gluey substance on the surface of its corolla. This species is found in the poorly studied High-Andean Paramo ecosystems of Colombia, Venezuela, Ecuador, and Peru and populations vary in the number of individuals with sticky vs. non-sticky flowers, making it also a great model for understanding the rarely tested ecological functions of stickiness in plants. Remarkably, stickiness in this species is present exclusively in flowers and not in other parts of the plant. Petals of sticky flowers are usually loaded with a diversity of trapped insects, which includes flies, bees, wasps, butterflies, and spiders, some of which could well function as pollinators (Kraemer 2001). However, the relatively large flowers are also attractive to herbivorous insects and different species of avian flower piercers of the genus *Diglossa* that are specialists in nectar-robbing. Here we use the natural polymorphism in the expression of floral stickiness as an example of a chemical-physical floral trait to focus on addressing the hypothesis that floral stickiness in *B. resinosa* functions as a defense against florivores. We use field surveys and manipulative experiments to measure the effects of stickiness on herbivory, asses the effects on plant fitness, identify factors that influence the defense-functionality of stickiness (context-dependency), and begin to identify the chemical properties of sticky and non-sticky *B. resinosa* flowers.

Materials and Methods

Bejaria resinosa Mutis ex L. (Ericaceae) is an up to 3 m tall shrub with red-purple flowers native to the Paramo and sub Paramo habitats in Venezuela, Ecuador, Perú, and Colombia between 1750 and 3700 m.a.s.l. (Matulevich Peláez et al. 2016). The most distinctive trait of this species is the stickiness in the flowers of some individuals (Melampy 1987; Kraemer 2001). The study was conducted in two populations of *B. resinosa* in the municipality of Sesquilé: Chorrera (CH), at 2800 m.a.s.l. and Tres Viejas (TV) at 3050 m.a.s.l., and a third population in the municipality of Sopó: Campo Alegre (CA), at 2700 m.a.s.l. in Cundinamarca, Colombia. The Flowers of the plant are visited by numerous species of insects, avian flower piercers (*Diglossa* spp.), and hummingbirds (Kraemer 2001; Rojas-Nossa 2007). The plant is highly self-compatible but requires pollinator visitation and the pollen has viscin threads that are thought to facilitate pollen transfer (Kraemer 2001).

Survey of floral damage and seed set

To determine if the stickiness affects the amount of damage on flowers by herbivores, we first surveyed sticky and non-sticky plants in each population, then randomly chose and marked one inflorescence per plant to count the number of flowers and buds and the proportion of those with any damage by floriferous species (rate of herbivory). One month later, we recorded the proportion of those flowers that had turned into fruit (fruit set) as a measure of fitness. In total, we recorded data from 50 plants in CH, 38 in TV, and 38 in CA. We found different proportions of sticky and non-sticky plants in the three populations (CH 2:3, TV 5:3, CA 4:1). During the survey, it became evident that much of the damage was due to a Tortricidae (Lepidoptera) larvae eating the anthers, style, and ovules of the flower, usually starting just before the anthesis of the flowers. So, we conducted a second survey to specifically estimate the amount of damage produced by the caterpillars in buds and flowers of sticky and non-sticky plants.

Statistical analyses were done using binomial regression with the total number of flowers or buds and the number of them with damage as dependent variable and stickiness and population as independent factors. Plant ID in each population was used as a random factor. If a significant treatment effect, were detected through the comparison of models with and without the fixed effect term, then we performed post hoc tests using the CLD function from the ‘emmeans’ in R.

Effect of stickiness on fruit set

To test for a causal relationship between stickiness, herbivory, and plant reproduction, we selected two inflorescences on each of ten different plants that produce sticky flowers. Just before anthesis, all of the buds of one of the inflorescences were each washed with 100 µl 95% methanol on a Q-Tip to remove the stickiness (**MeOH treatment)**; the buds on the other inflorescence were kept as a control (**Control treatment)** and 100 µl of MeOH was similarly applied to the pedicels as a sham control for the MeOH treatment. One week after the MeOH application (flowers in full bloom and just beginning to wilt) we assessed floral damage as the number of damaged flowers relative to the total number of flowers in each inflorescence. Inflorescences contained between 2 and 7 flowers. Fruit set was measured as the number of seed pods per initial number of flowers on each infructescence 100 days after the initial treatment. The data were analyzed by using binomial regression using the relative damage and relative fruit set as a response variable, treatment as an independent factor, and the individual plant as a random factor.

Effect of insects trapped on sticky petals on fruit set

To test for a potential proto-carnivorous, indirect defensive or pollinator-attracting function of stickiness, we selected two inflorescences on each of ten different plants producing sticky petals and applied one of two treatments: **No insects treatment**, insects were removed manually from each bud in the inflorescence without removing the stickiness; **Insect's treatment**, in this treatment the insects removed in the previous treatment were added for increased the number of insects trapped to each bud. We measured relative damage seven days and relative fruit set 100 days after the initial treatment, as described above. The data were analyzed by using binomial regression using the relative damage and relative fruit set as a response variable, treatment as an independent factor, and the individual plant as a random factor.

Hummingbird exclusion

Based on the findings in the first part of this study, we hypothesized that the population-specific differences in the effects of stickiness on florivory could be due to the differences in the presence of hummingbirds. Sticky plants are efficiently excluding interactions with insects both mutualist and antagonist. Thus, in populations with a high density of hummingbirds such as in the CA population, sticky plants will reap the benefits of protecting their flowers from herbivory without compromising pollination by hummingbirds. In such a population non-sticky plants will have lower levels of fruit set when florivorous are present. On the other hand, in a population with low densities of hummingbirds, sticky plants can be predicted to have a lower rate of pollination due to the additional reduction in pollination by insects. In this case, the costs of not attracting insect pollinators associated with stickiness are predicted to be larger than the benefits of protecting the flowers from florivorous. To test this hypothesis we chose eight sticky plants and eight non-sticky plants in the Tres Viejas population, on each plant two branches were selected. One of those branches was protected by a net with a mesh of 2 cm, supported on a wire structure that separated the net from the flowers. The holes allowed the major insect pollinators, bees, and bumblebees (Kraemer 2001), to visit the flowers but excluded the hummingbirds. Finally, we measured the relative fruit set of each branch by counting the number of flowers that became fruits on those branches. The results were analyzed by a binomial regression with fruit set as the dependent variable. Stickiness and the presence of the net were considered independent factors. Finally, the plant was considered a random factor. Effects of the factors were assessed by using a likelihood-ratio test, followed by a post hoc test using the CLD function from the 'emmeans' in R.

Chemical analysis

We collected volatiles following the headspace of flowers and leaves from sticky and non-sticky plants by enclosing them in 500 mL polyethylene cups fitted with ORBO-32 charcoal adsorbent tubes (Supelco, Bellefonte, PA, USA). Air was pulled through the cup at a flow rate of approximately 150 mL/min for 8 hours using an active air sampling vacuum pump (IONTIK, USA). Tubes were then capped and kept frozen before analysis. Before elution, we added 5 µL tetraline (90 ng/mL) as an internal standard to each tube. The tubes were then desorbed with 350 mL of dichloromethane and samples were analyzed in a Varian CP-3800 GC coupled to a Saturn 2200 MS and fit with a DB-WAX column (J&W Scientific) of 60 m × 0.25 mm id capillary column coated with polyethyleneglycol (0.25 mm film thickness). Helium (99.995%, Airgas®) was used as a carrier gas. Total ion chromatograms were integrated, and peak areas of individual compounds were normalized by the area of the internal standard. As before, we compared the changes in the compositions between sticky and non-sticky flowers and leaves using permutation analysis of variances (PERMANOVA) and a Nonmetric multidimensional scaling (NMDS) for visualization.

To analyze the non-volatile corolla surface chemistry, petals from both sticky and non-sticky plants were washed with Methanol to remove the sticky layer. These extracts were analyzed on an LC Dionex UltiMate 3000 (Thermo Scientific, Germering, Germany) equipped with a degassing unit, a gradient binary pump, an autosampler with 120-vial well-plate trays, and a thermostatically-controlled column compartment. Chromatographic separation was performed on a Hypersil GOLD aQ column (Thermo Scientific, Sunnyvale, CA, USA, 100 mm x 2.1 mm ID, 1.9 µm particle size). The flow rate of the mobile phase containing FA/water (A) and FA/acetonitrile (B) was 300 µL/min. A FA concentration of 0.2% v/v was used. The initial gradient condition was 100% A, changed linearly to 100% B in 8 min, maintained for 4 min, returned to 100% A in 1 min, and maintained for 3 min. The injection volume was 2 µL. The LC was connected to an Exactive Plus Orbitrap mass spectrometer (Thermo Scientific, Bremen, Germany) with a heated-electrospray ionization (HESI-II) source operated in the positive ion mode. The Vspray was set at 3.5 kV. The nebulizer and capillary temperatures were set at 350 and 320 °C, respectively; sheath gas and auxiliary gas (N_2) were adjusted to 40 and 10 arbitrary units, respectively. Nitrogen (>99%) was obtained from a generator (NM32LA, Peak Scientific, Scotland, UK). During the full scan MS, the Orbitrap-MS mass-resolution was set at 70000 (full-width-at-half-maximum, at *m/z* 200, R FWHM) with automatic gain control (AGC) target, $3x10^6$, C-trap maximum injection time, 200 ms, and a scan range of *m/z* 100–1000. The ions injected into the HCD-cell via the C-trap were fragmented with stepped-normalized collision energies of 10, 20, 30, and 40 eV. The mass spectra were recorded in the AIF (All-ion fragmentation) mode for each collision energy at R FWHM of 35000, AGC target, $3x10^6$, C-trap injection time, 50 ms, and a mass range of *m/z* 80–1000. The data obtained were analyzed using Thermo Xcalibur 3.1 software (Thermo Scientific, San Jose, CA, USA). With the relative peak area data, we compared the changes in the compositions between sticky and non-sticky flowers using permutation analysis of variances (PERMANOVA) and a Nonmetric multidimensional scaling (NMDS) for visualization. Moreover, we conducted a random forest (RF) analysis using the packages randomForest (Liaw and Wiener 2002) and varSelRF (Diaz-Uriarte 2015) in R version 3.3.1 (R Team Core 2021). To do this, we first conducted an RF classification analysis for sticky and non-sticky flowers. We then used 200 bootstrap iterations to select the compounds that best distinguished between sticky and non-sticky, followed by a t-test with the resulting compounds.

Results

Floral stickiness correlates with florivory and fruit set

The florivore damage to buds was strongly and consistently affected by floral stickiness and plant population (X^2 = 12.007, *df*= 3, *P* = 0.007; X^2 = 20.83, *df*= 4, *P* <0.001 respectively). Because stickiness did not equally reduce herbivory in all three populations, stickiness and population interacted as factors (X^2 = 6.1338, *df*= 2, *P*=0.046, Fig. 2.2A).

Stickiness and population both also affected the proportion of flowers damaged by florivores (X^2 = 23.82, *df*= 3, *P* <0.001; X^2 = 46.895, *df*= 4, *P* <0.001 respectively). Similar to the effects on buds, stickiness affected damage to open flowers differently in the different plant populations (X^2 = 15.989, *df*= 2, *P* <0.001. Fig. 2.2B).

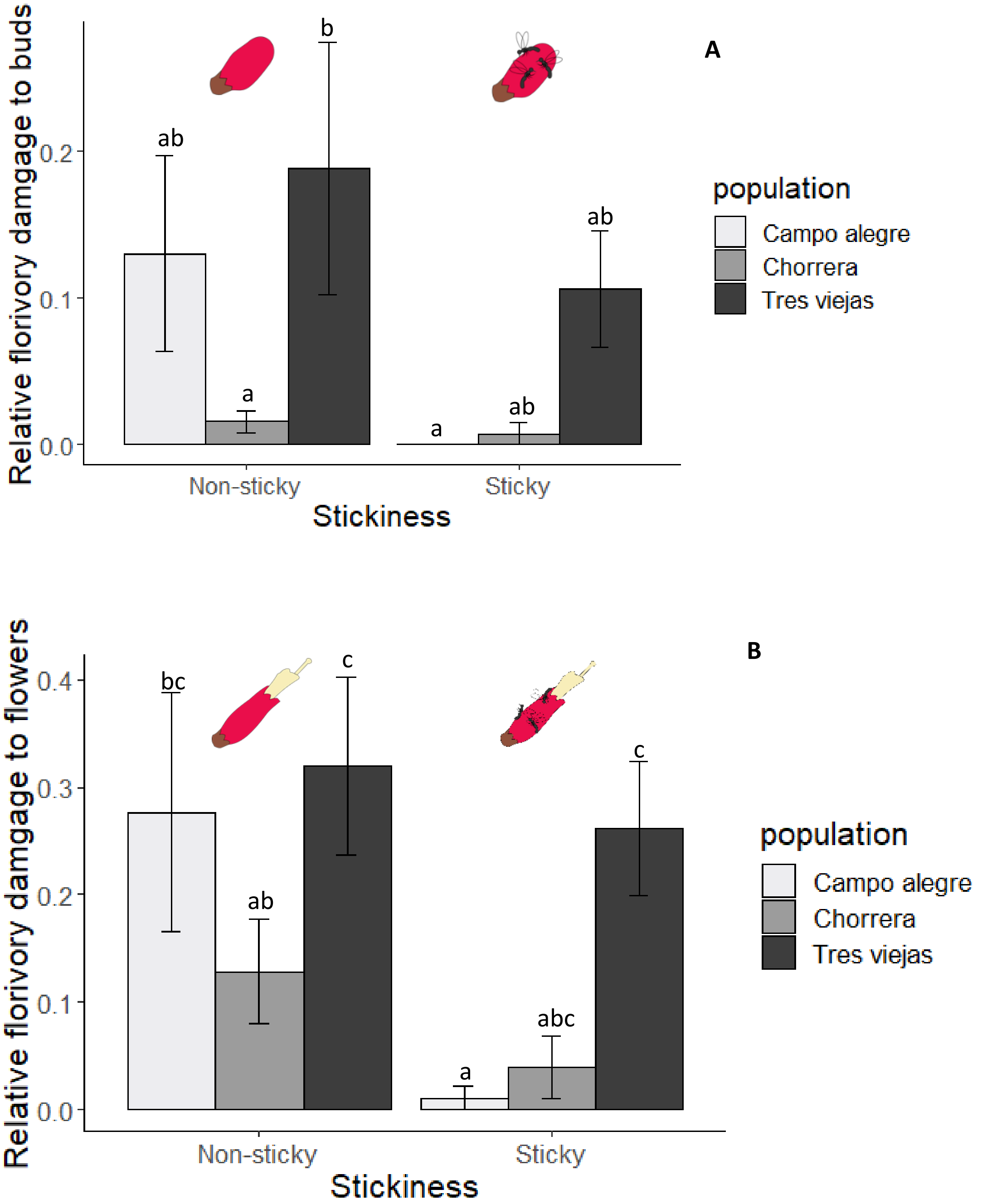

Figure 2.2. Mean relative proportion of buds (A) and flowers (B) (± SE) with damage by florivores in sticky and non-sticky plants of B. *resinosa* in three different populations. Different letters indicate significative differences (Tukey test, significance *P*=0.05).

Specifically, damage made specifically by florivore moth larvae was reduced in sticky buds (X^2 = 13.693, *df*= 3, *P* =0.003) and flowers (X^2 = 13.423, *df*= 3, *P* =0.003) with levels of damage varying between populations (buds: X^2 = 14.824, *df*= 4, *P* =0.005; flowers: X^2 =19.717, *df*= 4, *P* <0.001). However, here we did not observe a significant interaction between the stickiness and the population (buds: X^2 = 0.6961, *df*= 2, *P* =0.7, Fig. 2.3A; flowers: X^2 = 5, *df*= 2, *P* =0.081. Fig. 2.3B).

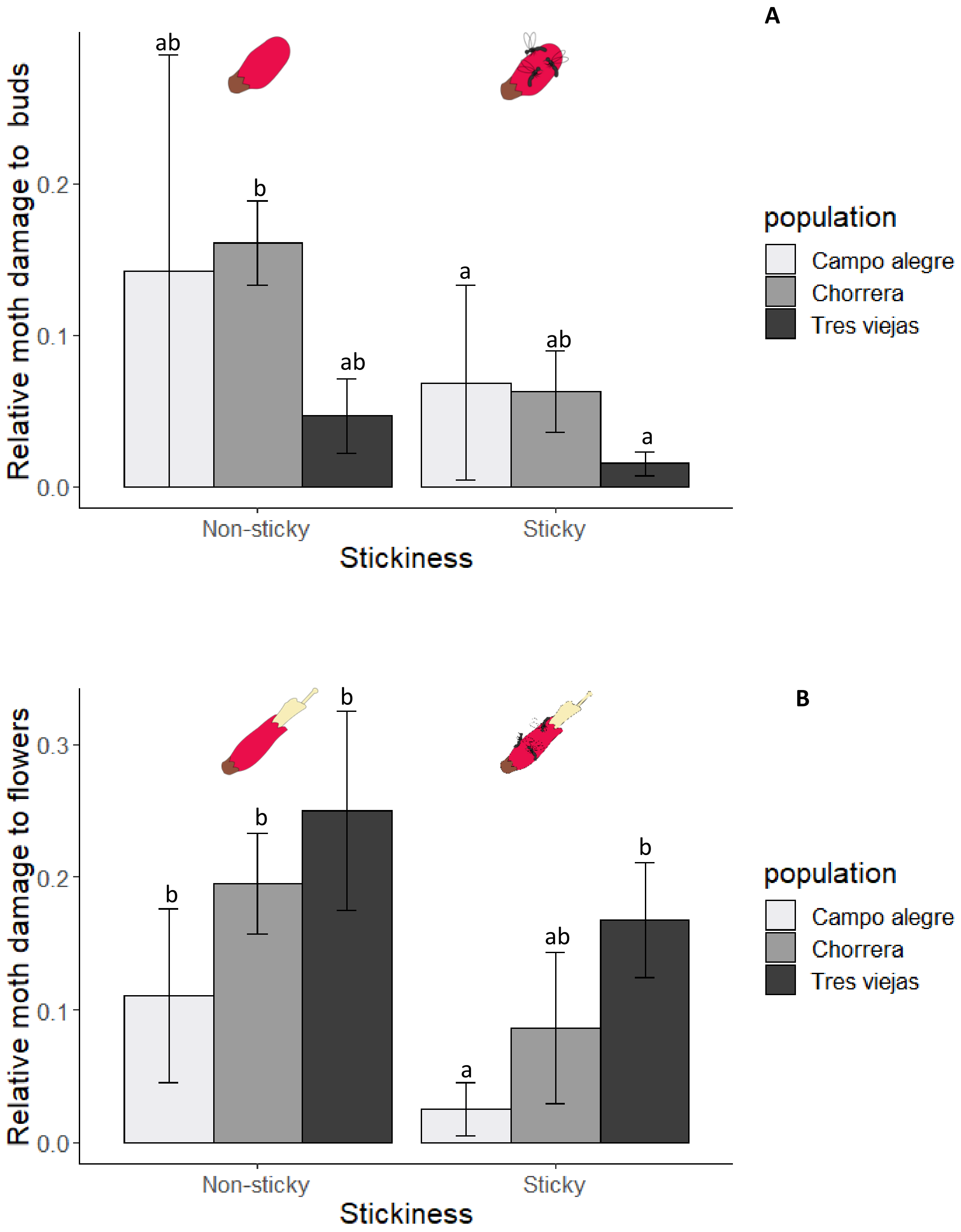

Figure 2.3. The relative amount of buds with damage by moth larvae (± SE) in sticky and non-sticky plants of *B. resinosa* in three different populations in buds (A) and flowers (B). Different letters indicate significative differences (Tukey test, significance *P*=0.05)

Most impactful, floral stickiness was associated with higher fruit set (X^2 = 29.511, *df*= 3, *P* <0.001) linking the negative effects of stickiness on florivory with a positive effect on fitness. As with florivory, seed set varied with plant population (X^2 = 26.89, *df*= 4, *P* <0.001). In consequence, floral stickiness and fruit set interacted statistically (stickiness x population: X^2 =17.761, *df*= 2, *P* <0.001. Fig. 2.4).

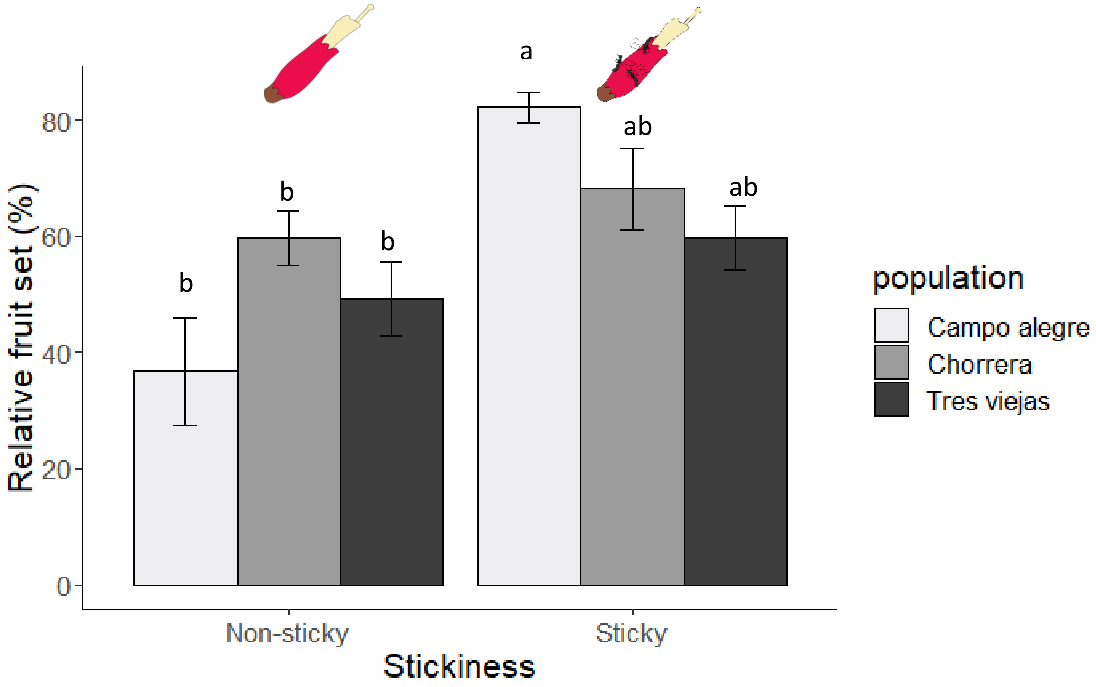

Figure 2.4. Mean relative fruit set (± SE) of sticky and non-sticky plants of *Bejaria resinosa* in three different populations. Different letters indicate significative differences (Tukey test, significance *P*=0.05)

Floral stickiness is causally linked to reduced florivory and increased fruit set in the field

In a field experiment manipulating the stickiness of phenotypically sticky flowers, none of the non-washed flowers suffered from any damage while 21% of the MeOH-washed flowers were attacked by herbivores on average. In consequence, MeOH-washed inflorescences had a 32.5% lower fruit set than non-washed control inflorescences (X^2=4.877, *df*=1, *P*=0.027. Fig. 2.5).

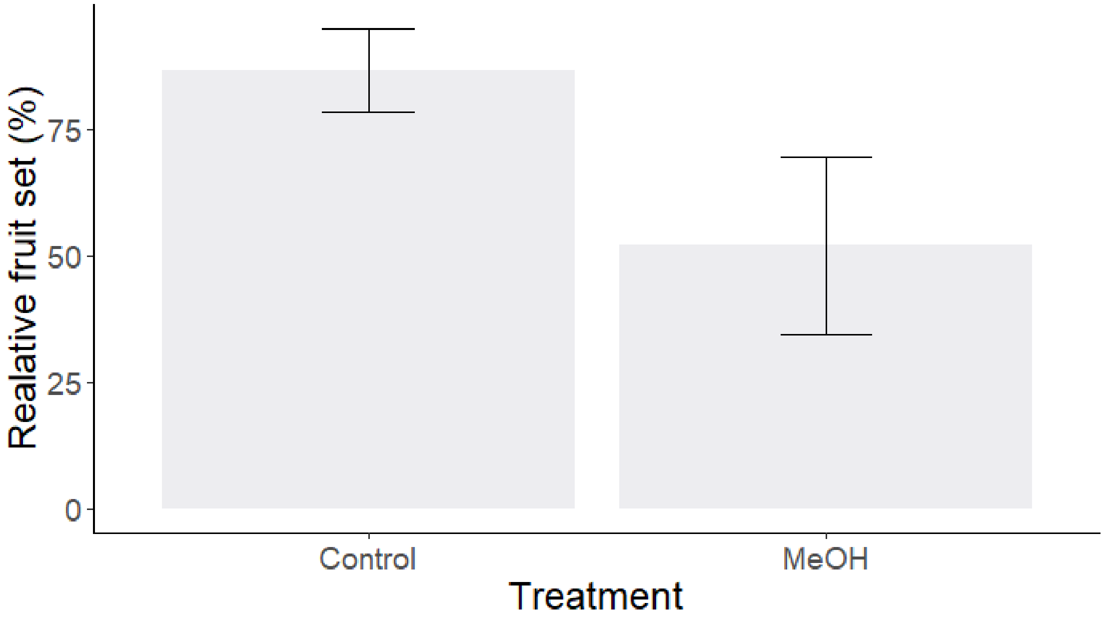

Figure 2.5. Mean relative fruit set (± SE) of sticky flowers of *Bejaria resinosa* washed with 100 µl of 95% methanol (MeOH) or with methanol added to the pedicel of the flower as a Control.

In addition to the field experiments, we conducted laboratory feeding assays with two generalist herbivore species to test for the actual feeding resistance rather than stickiness effect in the two different plant morphs There was no difference in the leaf area consumed by grasshoppers (*Bogotractis varicolor*) between petals from sticky and non-sticky flowers (*W* = 183.5, *P*=0.6651). However, snails (*Helix aspersa*) showed a preference to consume petals from sticky flowers (*W* = 59, *P*= 0.0001).

Trapped insects linked to higher fruit set

The presence of insects on the petals of sticky plants increased the fruit set relative to sticky flowers with no insects (X^2=5.4466, *df*=1, *P*=0.019, Fig. 2.6). We did not observe any kind of damage in either treatment.

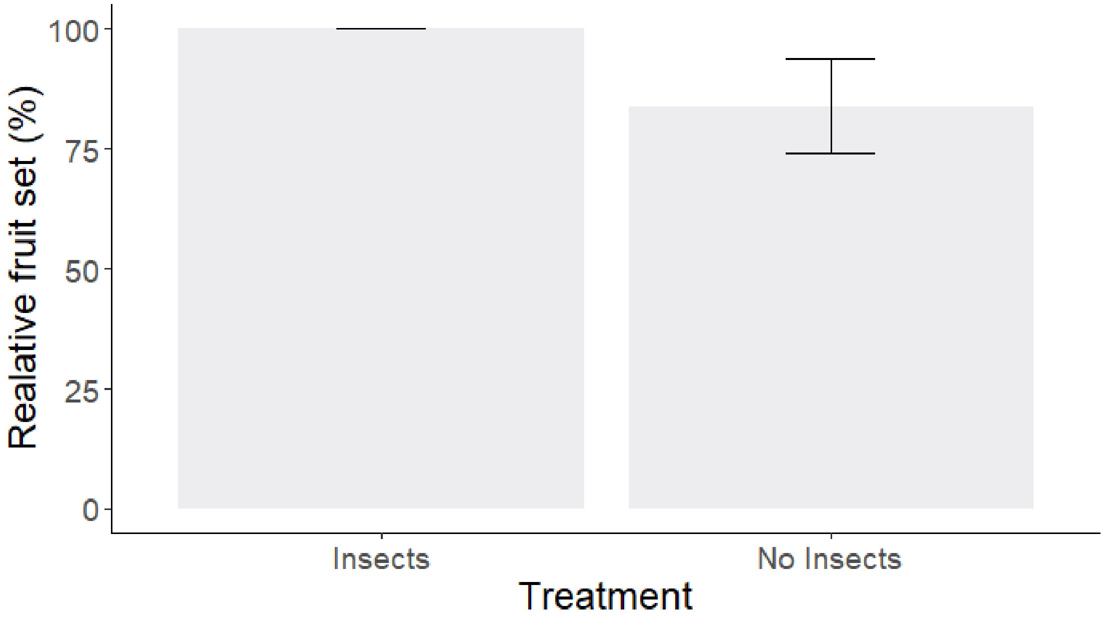

Figure 2.6. Mean (± SE) relative fruit set of flowers of *Bejaria resinosa* with two different treatments. **No Insects**, the insects stuck in these flowers were removed manually. **Insects** treatment, the insects that were removed from the *No Insect* treatment were added to this flower.

Bird exclusion with weak effects on fruit set

The presence of the net excluding hummingbirds did not affect fruit set (X^2=2.1817, *df*=2, *P*=0.3359), while stickiness did (X^2=5.944, *df*=2, *P*=0.051), and there was no interaction between stickiness and bird exclusion (X^2=1.45, *df*=2, *P*=0.22, Fig. 2.7).

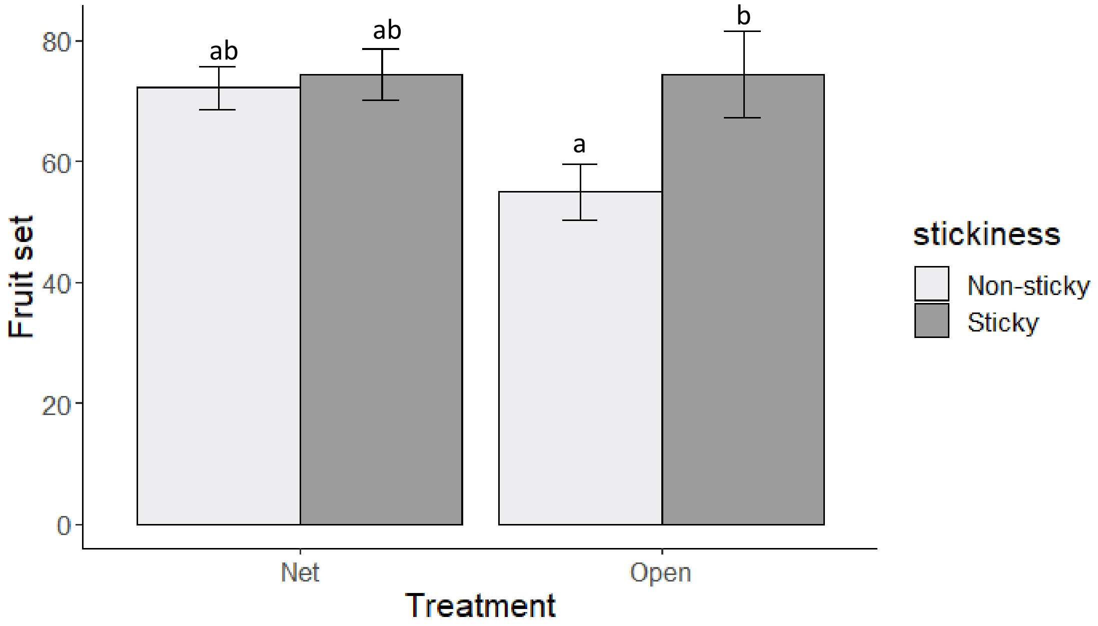

Figure 2.7. Mean of fruit set (± SE) of sticky and non-sticky inflorescences of Bejaria resinosa. Some inflorescences were covered by a net that allow bees and bumble bees to visit the flower but exclude hummingbirds and flower pierces, while others remain open to any flower visitor. Letter show significant differences (Tukey test, significance *P*=0.05).

Non-volatile chemistry differs between stickiness morphs

In general, emission of VOCs from flowers and leaves in both sticky and non-sticky plants was low, and there were no differences in VOC composition between flowers and leaves from sticky and non-sticky plants (Permanova: $F_{5,44}$= 0.7163, *P*= 0.6146, Fig. 2.8A).

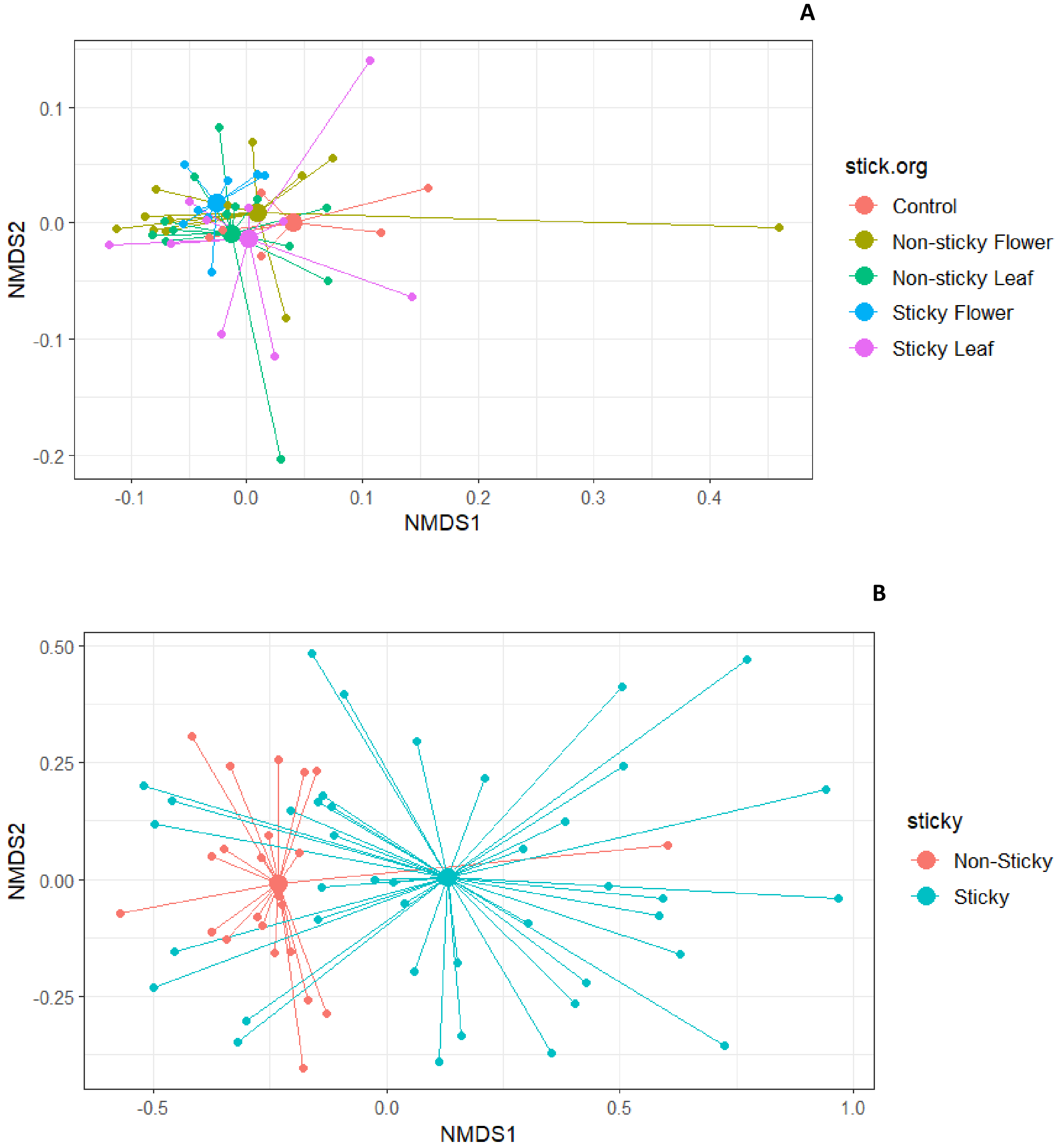

Figure 2.8. Non-metrical multidimensional scaling for **A.** volatile compounds from flowers and leaves and **B.** non-volatile compounds from sticky and non-sticky flowers of *Bejaria resinosa*

Twelve different non-volatile compounds were identified from petal extracts, which made up different chemistry in sticky and non-sticky flowers (PERMANOVA: $F_{1,62}$=10.864, P=0.001) (Fig. 2.8B). A subsequent random forest analysis (prediction error=0.1179) revealed two methoxyflavones (methoxyflavone1, methoxyflavone2) and one non-categorized compound as the three components best representing the differences between sticky and non-sticky flowers. Methoxyflavone2 and methoxyflavone1 were found in higher concentrations in the sticky flowers (t=-4.063, P<0.001 and t= -4.083, P<0.001), while the unidentified compound had higher concentrations in the non-sticky plants (t=2.348, P=0.022). The other ten compounds did not differ between sticky and non-sticky flowers.

Discussion

The production of sticky substances on the surface of leaf tissues, including sepals, has been hypothesized to have a variety of functions, among them: direct defense and indirect defense (LoPresti et al. 2018). Here we applied this hypothesis framework to floral tissues, which, unlike vegetative tissues, have evolved to mediate interactions with mutualists and are thus prone to expose the conflicts arising from defending against antagonists while still being able to attract mutualists (Lucas-Barbosa 2016; Kessler and Chautá 2020). Here we specifically focused on testing predictions of the direct defense hypothesis and probed for indirect defense hypotheses for the uncommon case that sticky substances were expressed on the petal surface. In support of the direct defense hypothesis, we found that stickiness was associated with reduced florivory and could be directly linked to increased reproductive success, however, these direct defenses varied across populations. Increased seed set of plants with sticky flowers in comparison to those with non-sticky flowers was mostly explained by the reduction of florivory from both mobile insects consuming petals and by more sessile moth caterpillars that consume reproductive structures (e.g. Anthers, style, and ovaries).

Thus, our data suggest that stickiness in reproductive tissues can serve the same defensive function as the stickiness of vegetative tissues despite the apparent conflict between defending against herbivores and attracting pollinators. Stickiness, in general, can mediate increased direct resistance against insect herbivores by causing insects to get stuck and die, reduce their performance, or repel them from landing on the plant altogether (Carter *et al.*, 1989. Van Dam & Hare, 1998). Repellency was clear in our experiments when flowers in which the stickiness was removed had higher levels of damage compared to wild-type sticky flowers. This repellency mechanism has been identified in other plant species. For example, different types of acyl sugars secreted from glandular trichomes of the wild tomato *Solanum pennellii* have been found to function synergistically to repel whiteflies and thrips (Leckie et al 2016). Acyl sugars seem to function without apparent toxicity but physically repel or immobilize attackers. Sticky glandular exudates of *Solanum lycopersicum* can trap and starve to death neonate caterpillars of *Helicoverpa armiguera* (Simmons et al. 2004).

In *B. resinosa* flowers, larvae of different moth species are the most damaging herbivores because they enter the flower buds and consume all the internal structures. Our data suggest that the physical adhesiveness may keep moths from ovipositing in the first place and caterpillars from moving from flower to flower (Fig. 2.9). This observation is consistent with findings on the sticky flowers of *Vriesea bituminosa* (Bromeliaceae), where 50 % of the trapped insects were phytophagous species (Monteiro and Macedo 2014), suggesting stickiness as an efficient trait limiting the number of interactors. For example, the sticky compounds could also be toxic (Carter *et al.*, 1989, Chatzivasileiadis & Sabelis, 1997) or deterrent (Avé et al. 1987). However, among the diverse arthropod community trapped on *V. bituminosa* as well as on *B. resinosa*, are still large numbers of species potentially beneficial to the plants. The resulting conflicting ecological effects are likely factors explaining the population differences in the stickiness effects on plant reproduction and are the likely force maintaining polymorphism in those populations.

Although plant stickiness was generally correlated with reduced herbivory and increased fruit set, these relationships varied significantly across plant populations. This variation could reflect differences in community composition and variable relative impacts of antagonists and mutualists affected by the defensive plant trait.

Figure 2.9. A. Moth larvae trapped on the sticky surface of petals of *Bejaria resinosa.* **B.** Flower showing the marks left after a visitation by the avian nectar robber *Diglossa* spp.

For example, in *Lycopersicon f. glabratum* exudating trichomes reduce predator mobility and also entrap small parasitoids (Kennedy 2003). *Bejaria resinosa* plants likely also suffer from this potential disadvantage. Many predators, such as spiders and parasitoid wasps as well as some potential insect pollinators, such as bees can be seen stuck to the flower petals (Fig. 2.1). Evaluating this potential ecological cost goes beyond the scope of this paper and requires a different experimental procedure than the one applied here. However, some of the results from our experiments give a clear indication as to where to focus the search for potential fitness trade-offs of stickiness in *B. resinosa*. For example, we applied a very simple field experiment to generally test for the indirect fitness effects of stickiness when insects are stuck to the petals. We found that sticky inflorescences with insects stuck to the petals had a 23.5% higher fruit production than those without insects. The experiment was deemed general (and thus also limited) in its approach because it allows for three mechanistic hypotheses for the apparent indirect effect of stickiness: A) the trapped arthropods attract predators that then also consume actively feeding florivores (indirect defense), B) the trapped organisms decompose and provide nutrients to the developing fruits/seeds (protocarnivory), or C) bird pollinators use the trapped arthropods as an additional nutritional resource and are those more attracted to these plants (increased pollinator reward). Because the actual damage levels between flowers with and without insects did not differ, the indirect defense mechanism (hypothesis A) is less likely to be an explanation for the observed fruit set pattern. The increased fruit set was not a result of indirectly reduced florivory. However, we can't fully dismiss an indirect defense function as a potential mechanism contributing to the population-specific outcomes of stickiness in *B. resinosa*. In other plant species, such as *Aquilegia eximia* (Ranunculaceae) and *Madia elegans* (Asteraceae) (Krimmel and Pearse 2013; LoPresti et al. 2015), the amount of carrion trapped on leaves and stems increased the number of predators attracted to the plants. Also, stickiness increased the efficacy of the predators *Pselliopus spinicollis* and *Hippodamia convergens* on *Heliothodes diminuta* and *Uroleucon madia* (Krimmel and Pearse 2014). In *B. resinosa*, we consistently observed a yet unidentified hymenopteran species foraging between the corpses on sticky flowers. So, there may be predators taking advantage of dead insects stuck to the plant, as has been seen in *Madia elegans* (Krimmel & Pearse 2013) and *Nicotiana attenuata* (Karban *et al.* 2019), where the amount of carrion increases the number of predators and guaranteed a higher fruit set.

Similarly, our results do not directly support a nutritional benefit for the plant from decaying arthropods (hypothesis B) as an explanation for the observed pattern in the fruit set. But the presence of carrion in the flowers can increase the number of natural enemies and therefore increase plant fitness as found in wild tobacco (Karban et al. 2019). Because of the potential of trapping large numbers of arthropods, stickiness is commonly associated with carnivorous plants such as *Drosera spp.* (Droseraceae), Nephentes spp (Nephentaceae), *Triphyophyllum peltatum*. (Dioncophyllaceae) and *Pinguicula* spp (Lentibulariaceae). Accordingly, carnivorous plants are thought to have evolved from plants that expressed some degree of stickiness, with an originally defensive function (Adlassnig et al. 2010). When plants become able to absorb some of the nutrients in the corpses, under poor nutrient conditions, natural selection can benefit plants with a higher rate of insect trapping and digestive ability (Ellison and Gotelli 2001; Adlassnig et al. 2010). Some non-carnivorous plant species (e.g. those that do not produce digestive enzymes) seem to gain nutritional benefits from the trapping of arthropods on otherwise defensive sticky surfaces. Plants like these are categorized as protocarnivorous and usually lack digestive enzymes (Givnish et al. 1984). Stickiness on floral tissues, like in *B. resinosa*, could potentially provide resources for the developing seeds. The results of our experiment adding insects to flowers indicate that *B. resinosa* has some degree of protocarnivory, as was for example found in *Geranium viscosissimum* leaves and stems (Spomer 1999). However, while nutrient uptake from decaying animals by leaves is probably more common than currently appreciated, to our knowledge there is no example for a plant being able to take up nutrients through their petals. Alternatively, insects stuck to leaves or flowers can also be a source of nutrients when the petals fall from the plant and decompose together with the insect cadavers (LoPresti et al. 2018). Again, this later mechanism is not likely to underlie the differences we observed in the manipulative experiment as it may not affect the actual fruit set at that time and as measured and because there is not enough time from anthesis of the manipulated flowers to their decomposition and utilization in plant metabolism to affect seed growth.

This is leaving interactions with other flower visitors (Hypothesis C), including pollinators as a potential mechanism for how arthropods stuck to the flower petals affect plant fitness. In *B. resinosa* specifically, hummingbirds are the major pollinators in the system and could use insects stuck to the flowers as an additional source of energy and protein. In this case, stickiness would provide a dual function as indirect defense and pollinator reward.

The hummingbird exclusion experiment showed a reduction in fruit set on the non-sticky inflorescences that were open to the visitation of any pollination mirroring the general differences we found between sticky and non-sticky plants in the other experiments. However, there was no reduction in fruit set when hummingbirds were excluded, rejecting the hypothesis that hummingbirds could be a major indirect factor explaining stickiness-mediated variation between populations. However, this specific result of a relative increase in fitness in non-sticky plants, when birds are excluded, points to yet another potential factor: nectar-robbing flower piercers (*Diglossa* spp.). Our exclusion experiment excluded all birds, including those with potential antagonist effects. As primary robbers, flower piercers could directly affect plant fitness or through facilitating secondary nectar robbers by providing initial access wounds to the flowers (Fig. 2.9B)(Maloof and Inouye 2000). Sticky substances in plants are usually complex compound mixtures (Asai et al. 2010, 2012; Ohkawa et al. 2012) with many of the components having some antibiotic, specifically antimicrobial, properties (Omosa et al. 2014). Thus, the stickiness can provide a mechanism to persistently cover surfaces with antimicrobial substances and so work as a barrier that can prevent secondary infection in the holes that have been opened by other organisms, such as nectar robbers. Given the high prevalence of damage by avian flower piercers in *B. resinosa*, stickiness could indeed provide a major mechanism to prevent secondary infections with plant pathogens.

The interactions between the plant and other organisms are thought to be largely mediated by plant secondary metabolism. Plant chemistry determines which organisms can interact by providing information before an organism touches the plant tissue (e.g. VOCs, color pigments) and when it touches or manipulates the plant tissue (e.g. toxins, deterrents, detractants, but also VOCs)(Kessler 2015). The VOC emissions from flowers and leaves of *B. resinosa* are relatively low and do not differentiate between tissues and phenotypes. Low floral VOC emissions are usual in plants with ornithophiles syndromes of pollination, since the main signal for pollination attraction is not olfactory but visual (Knudsen et al. 2004). Moreover, the similarity in VOC emission between sticky and non-sticky flowers also suggests that *B. resinosa* is not advertising its sticky surfaces to oncoming insects, thus neither repels (chemical aposematism)(Pearse et al. 2013) or attracts arthropods to its sticky surfaces (VOC attraction) via VOC cues (El-Sayed et al. 2016).

In contrast to the volatile compound production, non-volatile chemistry varied strongly between sticky and non-sticky flowers. The preliminary chemical analysis we present with this study is meant to establish if differences in stickiness between plant phenotypes can be explained by differences in surface chemistry and so begin to close a large knowledge gap on the chemistry of stickiness (Adlassnig *et al*. 2010). The major chemical components we found in far higher concentrations on the surface of sticky *B. resinosa* flowers, methoxyflavones, seem to be commonly associated with sticky plants (Urzua and Cuadra 1990; Greenaway et al. 1992; Omosa et al. 2014) and have reported antifungal activity (Omosa et al. 2014). Interestingly, experiments with generalist herbivores, in which grasshoppers and snails were fed on petals in isolation from the plant and thus relatively unaffected by stickiness suggested a higher herbivore resistance of non-sticky floral tissue.

The increased feeding on sticky petals relative to non-sticky petals by the snails is opposite to the herbivore assays in the field. On one hand, this difference can point to a mainly deterrent, non-toxic nature of the sticky compound mixture on the surfaces of *B. resinosa* petals similar to that mediated by acyls sugars (Leckie et al. 2016; Ben-Mahmoud et al. 2018) and the potential presence of actually higher concentrations of toxic or anti-digestive compounds in tissues of non-sticky petals. The expression of one not yet identified compound found in the non-sticky petals follows exactly that pattern and could have a deterrent effect on the snails specifically as well as other herbivores. On the other hand, snails may prefer some of the chemistry associated with stickiness. In conjunction with metoxyflavones, terpenes are also very frequent on sticky surfaces (Midiwo et al. 2002; Drewes et al. 2006; Jiménez-Pomárico et al. 2019), and previous studies have found that *Helix aspersa* preferentially feeds on terpene-rich tissues (Linhart and Thompson 2021). So it is possible that the stickiness that frequently functions as a defense against most herbivores, in the case of snails is being used as an attractant. In conclusion, the chemical differences in petal surface chemistry observed in this study explain the differences in florivory between the two different stickiness morphs of *B. resinosa*. The understanding of what makes a mixture of compounds sticky, and the physical properties of individual compounds and mixtures goes beyond the scope of this paper. We, however, still try to thoroughly report the chemical differences to move towards that kind of understanding.

Although more targeted experiments on the indirect effects of stickiness and the chemical mechanisms are needed, our findings nevertheless indicate that direct and indirect ecological effects of stickiness negotiate the outcome of the conflict between attracting friends while repelling foes and thus create the apparent population context-dependency.

Understanding the ecological conflicts between defending against antagonists while attracting mutualists, specifically with chemical traits in floral tissues, has become a major focus of chemical ecology research in recent years and has been viewed as crucial in understanding the evolution of floral traits (Kessler and Halitschke 2009; Sletvold et al. 2015; Ramos and Schiestl 2019). Tentative indication for the existence of such ecological conflicts in *B. resinosa* is apparent in the context-dependency of the direct defensive effects of stickiness.

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Chapter 3

Floral stickiness, a potent chemical resistance trait against primary and secondary nectar robbers

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Abstract

Many flowering plant species produce nectar as a reward for the organisms that pollinate or protect them from antagonists. However, these nectar rewards can also be attractive to other organisms that may have neutral or even negative effects on plant fitness. Thus, plants negotiate an evolutionary compromise to defend against antagonists (e.g. herbivores, nectar robbers), while still being able to attract mutualists (e.g. pollinators, natural enemies of herbivores). Among the most effective chemical defenses are sticky surfaces that are toxic or reduce the movement of antagonists. However, stickiness in flowers is not a common trait likely because of the apparent conflict between defense and pollinator attraction. *Bejaria resinosa* is a plant species that is dimorphic for sticky petals in its native High-Andean forest habitats. Among a diverse community of interactors, this plant is frequently visited by primary and secondary nectar robbers. Here we test the hypothesis that stickiness in flowers of *Bejaria resinosa* functions to reduce visitation by primary and secondary nectar robbers. We evaluate the proportion of damage by primary robbers and levels of visitation of secondary nectar robbers. Moreover, we simulate nectar removal to measure the effect of nectar robbers on fruit set. The extent of floral damage by primary nectar robbers varied with plant population but was not generally affected by the stickiness of the flowers. However, secondary nectar robbers, which are mostly insects, visited sticky flowers less frequently than non-sticky flowers. Interestingly, the fruit set of simulated robbed flowers was not different from those not robbed. These results show floral stickiness in *B. resinosa* mediates resistance against nectar robbers, but the fitness outcome of the resulting interactions is context-dependent.

Key words: Diglossa, hummingbirds, Andes mountains, context-dependent.

Introduction

Nectar is a sugar-rich solution produced by specialized plant tissues in flowers or on leaves and stems to facilitate interactions with mutualists, such as pollinators to aid in fertilization and outcrossing (Heil 2011), or predators, mediating indirect defenses (Kessler and Heil 2011). Concerning pollination, floral morphology, chemistry, and reward accessibility are thought to be under natural selection to optimize the interaction with the most efficient pollinators (e.g. pollinator fidelity hypothesis) (Thøstesen and Olesen 1996; Adler 2000; Parachnowitsch et al. 2019) and to minimize interactions with less efficient pollinators, pathogens, and florivores (e.g. floral defense hypothesis) (Adler 2000). Moreover, floral rewards, such as nectar, pollen, and oils provide attractive energy and nutrient sources and are thus commonly sought after by organisms not providing pollination services. This robbing of floral rewards can be understood as parasitism on the mutualistic interaction between pollinators and plants. Similar to other parasitisms of mutualisms, such as myrmecophiles parasitizing ant-plant mutualisms (Whitehead et al. 2014) or the attraction of hyperparasitoids to plant-parasitoid indirect defense mutualisms (Poelman et al. 2012), floral reward robbers can have a diminishing effect on plant fitness.

Nectar robbing is likely the most common type of parasitism on floral rewards. Significant costs of nectar robbing can arise from increased plant resource expenditure without pollination (production costs) and reduced attractiveness of the flowers to pollinators due to reduced rewards (ecological costs) (Irwin and Brody 1998; Varma et al. 2020). Depending on their ability to open access points in the flower to reach a nectar source, nectar robbers have been divided into primary and secondary nectar robbers. Primary nectar robbers are species that can actively overcome plant barriers that limit access to nectar. Most commonly nectar robbers may open a hole in the side of a flower to obtain nectar, thereby avoiding using the functional flower openings used by pollinators (Inouye 1980; Irwin et al. 2010) and thus do not come into contact with the reproductive organs. Such robbers include short-tonged bees and some species of bumblebees, as well as birds (Inouye 1983). Among the avian nectar robbers, the most notorious in the neotropics are flower piercers of the Genus *Diglossa*, who have evolved specialized bill morphologies to open holes in the flowers to take the nectar (Schondube and Del Rio 2003; Schondube and Martinez Del Rio 2004). In contrast, secondary nectar robbers cannot access the nectar themselves but opportunistically use the holes already opened by primary nectar robbers. Secondary nectar robbers can include a diversity of species of broad taxonomic origin, such as insects, birds, and mammals (Irwin et al. 2010). Nevertheless, like primary nectar robbers, secondary nectar robbers also do not provide any pollination services to the plant and thus are functionally parasites of the plant-pollinator mutualism (Inouye 1980; Irwin et al. 2010; Cuevas and Rosas-Guerrero 2016; Varma et al. 2020).

Due to its antagonistic, parasitic nature nectar robbing can have strong negative effects quite like predation and competition. And, like with predation and competition, the outcome of a nectar robber-plant interactions may depend on the interplay between the supply of resources and the density responses of the consumer (Chamberlain and Holland 2008). In consequence, these interactions can have negative, positive, or neutral outcomes for plant fitness (Maloof and Inouye 2000). Negative effects are more likely to be observed when I) the presence of nectar robbers is directly deterrent to the main pollinators (Roubik 1982), II) the removal of nectar by robbers increases the resource investment into nectar production, relative to that into seed growth (Pyke 1991), III) the nectar robbers damage the reproductive organs (Irwin et al. 2010), or IV) the pollinators avoid robbed plants for the lack of a reward (Irwin and Brody 1998). Positive effects can be observed when the reduction in the amount of nectar by nectar robbers increases the movement of pollinators and so increases visitation and outcrossing (Maloof 2001). If the effects of nectar robbers are mainly negative, they provide a strong agent of selection driving the evolution of protective mechanisms (Murphy and Breed 2008).

Several traits have been described that are thought to counteract the presence of nectar robbers (Inouye 1980). For example, the presence of extrafloral nectaries to distract ants from visiting the flowers (Wagner and Kay 2002), the thickening of petals to make the perforation more difficult (Inouye 1983; Murphy and Breed 2008), the production of dense inflorescences that deny access to the flowers (Barrow 1976; Murphy and Breed 2008) or the inclusion of chemical deterrents into the nectar or the production of specific deterrent floral volatiles (Stephenson 1982; Junker and Blüthgen 2008). Due to the hypothesized high ecological and metabolic costs of producing chemical defenses in reward tissues, they are predicted to be more frequent in long-lived flowers with substantial quantities of nectar and specialized pollination systems (Inouye 1983). One chemical trait with a predominantly defensive function is the secretion of sticky, adhesive compounds onto the surface of plant tissues. Likely because of the high deterrence efficiency of sticky compounds, stickiness is rarely observed in flowers, and its effects on pollinators, florivores, and nectar robbers are poorly understood (e.g. Krimmel & Wheeler 2015, Monteiro & Macedo 2014). But it is also because of this efficiency as a defense that floral stickiness provides an excellent model to study the ecology and evolution of conflicting interactions between plant mutualists and antagonists mediated by plant chemistry. (McCarren et al. 2021). Many hypotheses have been proposed for the function of sticky flowers such as limiting water loss, protocarnivory, indirect defenses, and prevention of nectar robbing (Vlok and Schutte-Vlok 2003; McCarren et al. 2021), but few have directly been addressed.

Bejaria resinosa Mutis ex L. (Ericaceae) is a self-compatible High-Andean shrub that is dimorphic for the expression of sticky substances on the petal surface. Previous studies have demonstrated that this kind of stickiness reduces the damage of reproductive tissues by florivores (Chautá, et al in rev.). During these initial experiments, it became apparent that nectar robbing on *B. resinosa* flowers by *Diglossa* spp. birds are very common and attract secondary nectar robbers as well, suggesting negative fitness effects that rival those of florivory. However, the effect of primary and secondary nectar robbers on plant performance and the role of petal stickiness in mediating resistance to those nectar robbers is unknown. Through field surveys and manipulative experiments, we test the hypothesis that floral stickiness can function as a defense against primary and secondary nectar robbers. We predict that stickiness affects the behavior of primary and secondary nectar robbers and so influences pollination success and fruit set.

Materials and Methods

The system

Bejaria resinosa Mutis ex L. (Ericaceae) is a shrub that can grow up to 3 m tall, with red-purple flowers. It is commonly found in paramo and sub-paramo habitats in Venezuela, Ecuador, Perú, and Colombia between 1750 and 3700 m.a.s.l. (Matulevich Peláez et al. 2016). (Kraemer 2001). The species is dimorphic for stickiness on the exposed side of the petals, with the proportion of individuals expressing sticky flowers varying by population (in rev.). The study was conducted in the Department of Cundinamarca, Colombia, in two populations of *B. resinosa* in the municipality of Sesquilé: Chorrera (CH, 5°02'56.68'' N, 73°46'45.87'' W, at 2800 m.a.s.l.) and Tres Viejas (TV, 5°0.2'40.92''N, 73°46'25.59'' W at 3050 m.a.s.l.) and one more population in the municipality of Sopó: Campo Alegre (CA, 4°54'29.11'' N, 73°59'16.78'' W at 2700 m.a.s.l. The Flowers of *B. resinosa* are visited by numerous species of insects and hummingbirds (Kraemer 2001), although the floral morphology (e.g. large red-purple flowers) and scent emission (e.g. very low emissions) tightly match predictions of an avian pollination syndrome. The damage by specialized nectar robbers (Genus *Diglossa*) is very common in this species (Rojas-Nossa 2007; Rojas-Nossa et al. 2016). In the study area the species *Diglossa humeralis* (Fig. 3.1A), *D. cianea*, *D. albilatera,* and *D. citoides* are frequently observed as primary nectar robbers and bees and bumblebees as secondary nectar robbers (Chautá per. obs). The plant is self-compatible yet not autonomous and the pollen grains are grouped by viscin threads, which is thought to facilitate pollen transfer under pollinator-limited conditions (Kraemer 2001).

Effect of stickiness on primary nectar robbers

To determine if the stickiness affects the attack frequency of flowers by *Diglossa* spp. flower piercers, we first surveyed sticky and non-sticky plants in each population, then randomly choose and marked one inflorescence per plant to count the number of flowers and buds and the proportion of those with any damage by *Diglossa* piercers (rate of nectar robbing) (Fig. 3.1B). After one month, we recorded the proportion of flowers that eventually became fruits (fruit set) as a proxy measure of fitness. In total, we recorded information from 50 plants in CH, 38 in TV, and 38 in CA. We found different proportions of sticky and non-sticky plants in the three populations (CH 2:3, TV 5:3, CA 4:1). Statistical analyses were done by using binomial regression with the total number of flowers or buds and the number of them with damage as dependent variable and stickiness and population as independent factors. Plant identity within each population was used as a random factor. If a significant treatment effect was found with a likelihood-ratio test, we performed post hoc tests using the CLD function from the ‘emmeans’ in R (R Team Core 2021)

Effect of stickiness on secondary nectar robbers

To understand the role of stickiness in preventing the stealing of nectar by secondary nectar robbers we choose one flower with damage by flower piercers per plant and recorded the number of secondary nectar robbers that successfully use the hole to take nectar for 15 min. There were always two observers, one observing a sticky and the other a non-sticky flower on a neighboring plant of the opposite phenotype. The observations were made in the morning from 8 am to noon and then from 2 pm to 5 pm. Due to the high number of zeros in the data, we used a zero-inflated Poisson regression using the package pscl in R for the statistical analysis. Thereby we used the number of secondary nectar robbers visiting as a dependent variable and stickiness (sticky or not sticky) as an independent treatment factor.

Effect of nectar robbing on fruit set

To test if secondary nectar robbers have any differential effects on fruit set of sticky and non-sticky plants, we chose and labeled with a colored sewing threat, six recently opened flowers per plant, which were assigned to three different treatments: control, nectar robber, or secondary nectar robber. In the control treatment, the base of two flowers was covered with clear tape to prevent damage by flower piercers. In the nectar robber treatment, we open a hole in the base of the corolla of two flowers using a toothpick to simulate the opening usually made by the flower piercers. In the secondary nectar robber treatment, we opened a hole at the base of the corolla as in the previous treatment, and in addition, three times a day (8 am, 12m, and 4 pm) removed the nectar by using glass capillaries, to simulate nectar removal by nectar robbers. This nectar removal was maintained until flower senescence (approx. 12 days). In each treatment, one of the flowers was supplemented with pollen from a neighboring plant using a small brush, while the other, paired flower was not supplemented with pollen. This allows us to evaluate pollen limitations on each treatment. One month after the end of the treatments we counted the proportion of flowers that became fruits (fruit set). The results were analyzed by a binomial regression, using the libraries lme4 and emmeans in R. Treatment and stickiness were included as a fixed effect, and the plant was included as a random effect. Significant effects of treatment on fruit set were detected through the comparison of models with and without the fixed effect term.

Figure 3.1. Major nectar robbers associated with *B. resinosa.* **A.** *Diglossa humeralis,* one of the species of flower piercers that visit *B. resinosa* (Photo: Nicolás Rozo-Pinilla). **B.** Arrows pointing to the characteristic marks open by *Diglossa* spp., the primary nectar robbers of *B. resinosa*. **C.** honeybee stick to a flower of *B. resinosa*, when trying to rob nectar through the hole opened by *Diglossa* spp. **D.** *Bombus robustus* stealing nectar from a non-sticky flower.

Results

Effect of stickiness on primary nectar robbers

The effect of stickiness on damage to the flower buds before anthesis by flower piercers was highly variable and thus did not show a consistent pattern (X2 = 2.319, df= 3, P = 0.5). Likewise, the proportion of buds attacked by flower piercers did not vary with plant population (X2 = 6.313, df= 4, P = 0.177) and we did not observe an interaction between the effects of stickiness and population (X2 = 1.591, df= 2, P = 0.4513. Fig. 3.2A).

This overall pattern was different in fully developed flowers post-anthesis. There was a consistent effect of stickiness (X2 = 10.454, df= 3, P = 0.015) on *Diglossa* damage to flowers during anthesis. The proportion of mature flowers damaged by flower piercers varied with plant population (X2 = 36.614, df= 4, P <0.001), with an interaction between the effects of stickiness and population (X2 = 7.64, df= 2, P =0.022, Fig. 3.2B).

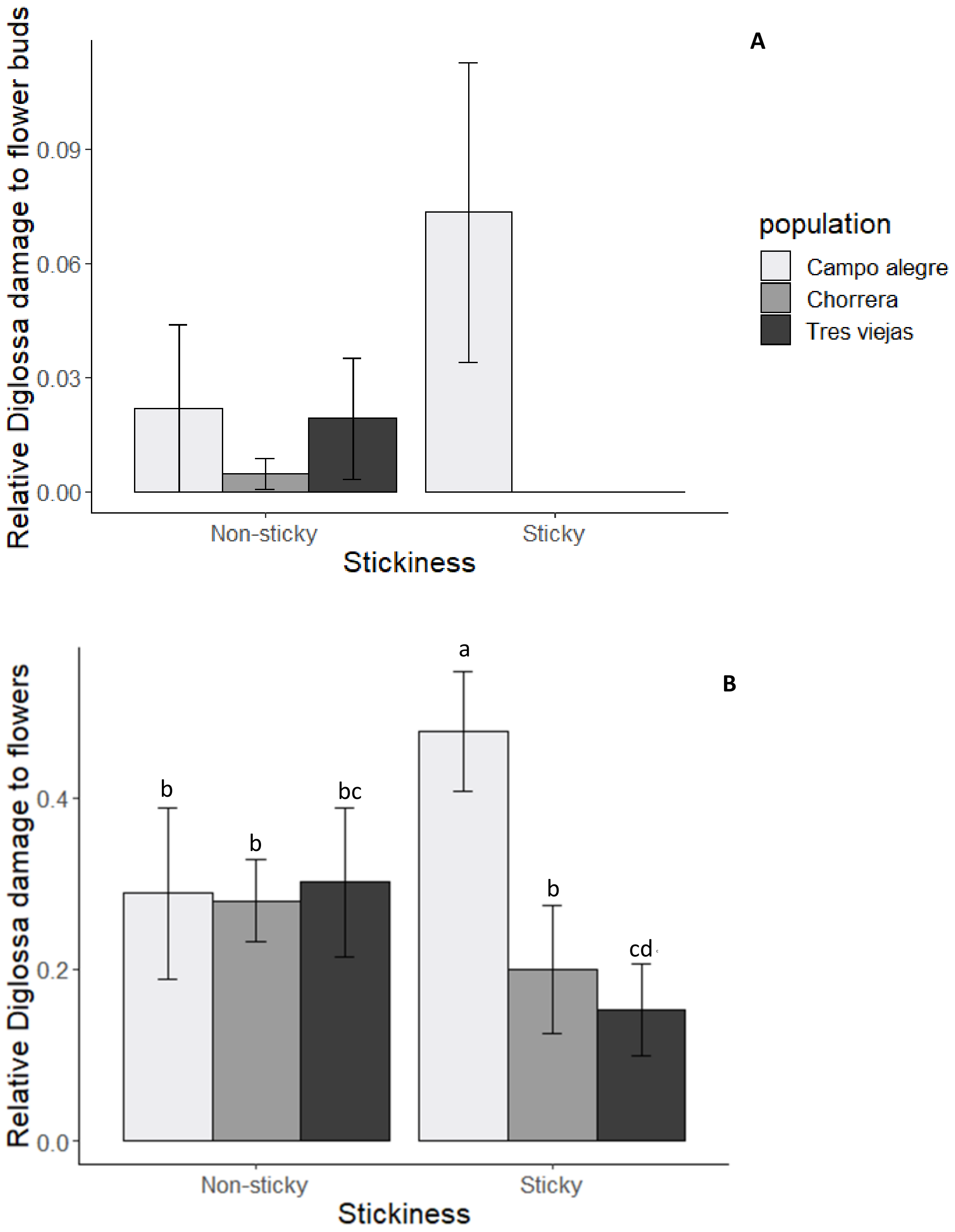

Figure 3.2. Mean proportion (± SE) of flower buds before anthesis (A) and open flowers (B) with damage by flower piercers of the genus *Diglossa* on sticky and non-sticky plants of B. *resinosa* in

three different populations. Different letters indicate significative differences (Tukey test, significance *P*=0.05).

Effect of stickiness on secondary nectar robbers

The field observation shows a reduction of secondary nectar robbers visiting sticky in comparison to non-sticky flowers post-anthesis (z=2.725, df=4, p=0.006. Fig. 3.3). The most frequent secondary nectar robbers were *Apis mellifera* and the bumblebees *Bombus atratus* and *Bombus robustus*, and less frequently, the hummingbird *Colibri coruscans*. As an interesting natural history note, we observed some individual bees and bumblebees that had learned to land on the exerted anthers of *B. resinosa* to avoid the stickiness and so became potential pollinators instead of nectar robbers. However, this behavior was relatively rare (9.23% of observed bee visits).

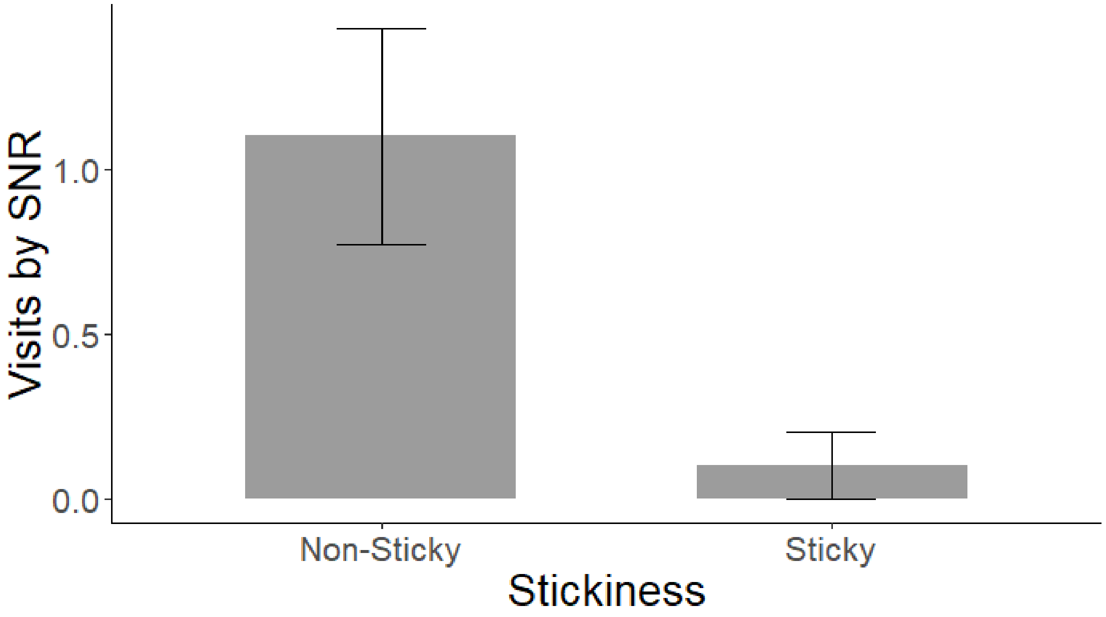

Figure 3.3. Mean(±SE) number of visits by secondary nectar robbers (SNR) to sticky and non-sticky flowers of *Bejaria resinosa* with holes open by flower piercers (*Diglossa* spp.).

Effect of nectar robbing on fruit set.

Although stickiness strongly affected the behavior of primary and secondary nectar robbers, the different nectar robbing manipulations did not affect fruit set (X2=10.394, df=6, p=0.109), while the effect of stickiness on fruit set was marginally significant (X2=16.749, df=10, p=0.08) with no interaction between treatment and stickiness (X2=8.9319, df=5, p=0.118. Fig 3.4).

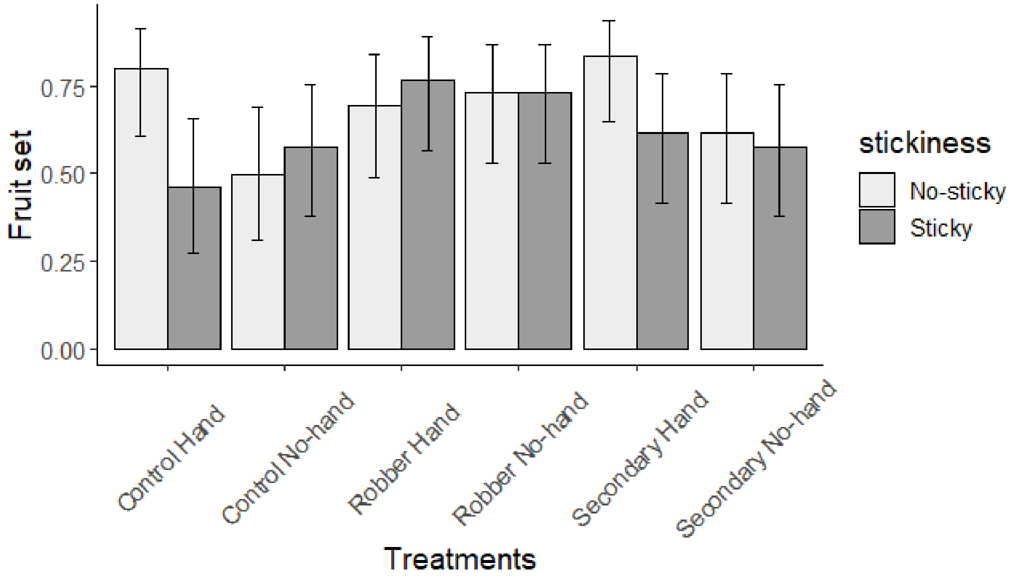

Figure 3.4. Mean (±SE) fruit set of sticky and non-sticky flowers under three different treatments. **Control**, the base of the flower was covered with clear tape to prevent damage by flower piercers. **Robber**, we opened a hole artificially to mimic damage done by flower piercer (*Diglossa* spp.), which allows secondary nectar robbers to access nectar through that hole. **Secondary nectar robber**, in addition to artificially punching a hole, we removed the nectar from the flowers three times a day.

Discussion

Our results indicate that floral stickiness influences the behavior of primary and secondary nectar robbers, with the outcome of the interaction varying with the population context. However, there is no evidence of a consistent effect of nectar robbers on *B. resinosa* female fitness.

Primary nectar robbers are affected by sticky petals

Changes in the levels of nectar robbing in different populations have been found in other systems (Guitián et al. 1994; Morris 1996; Irwin and Maloof 2002). The interaction between stickiness and population is likely to reflect the differences in community composition. For example, the presence of other plants with flowers can attract the robbers and reduce robbing pressure on a focal plant species (associational effects; Irwin, Brody & Waser 2001), or the ratio of pollinators to robbers could change dramatically and change the magnitude and directionality of a trait effect on seed set (Arizmendi 2001). For the *B. resinosa* study system this would mean that when nectar is abundantly available in the community and/or the ratio between pollinators/flower piercers is low, flowers piercers may avoid sticky flowers because the adhesive compounds could soil their beaks and make it harder to manipulate and pierce other flowers or cause them to commit more time to clean. In turn, under nectar-limited circumstances, these increased handling costs, associated with collecting nectar from sticky flowers may be worth paying for the nectar robbers. Such context-dependency of foraging preferences is also known from pollinators. For example, *Apis mellifera*, chooses artificial flowers with different handling costs depending on the context of the relative food quality of additional flowers available in the community (Shafir et al. 2002). Here we demonstrated a generally repellent effect of floral stickiness to *Diglossa* spp. nectar robbers, but our data also clearly suggest that the magnitude of the effect can vary with the population sampled. Future studies should test for environmental factors, such as relative nectar availability (plant community composition) and competition with other nectar consumers (pollinator and nectar community composition) that may affect the distribution of damage by nectar robbers in this context-dependent interaction.

Secondary nectar robbers reduce visitation to sticky flowers

Non-sticky flowers of *B. resinosa*, were more vulnerable to arthropod secondary nectar robbers than sticky flowers. This is likely because the stickiness makes it harder for the insects to walk over the petal and reach the incision created by the primary nectar robbers. Although this study confirmed that some individual bees are capable to land on the anthers, thereby avoiding the sticky petals and consequently can pollinate the flowers, they seem to prefer to rob nectar through the *Diglossa*'s incision holes. The specific morphology of the flowers in combination with the petal stickiness makes nectar robbing energetically easier than entering the flower through the top opening (Fig. 3.1). A similar optimal foraging mechanism was observed in the taxonomically related and morphologically similar *Vaccinium ashei*, where the net energy gain per second for the secondary nectar robber was higher when robbing compared to entering the flower through the corolla opening (Dedej and Delaplane 2005). Other plant species with sticky flowers such as some species of the genus *Erica* (McCarren et al. 2021), or the bromelia *Vriesea bituminosa* (Monteiro and Macedo 2014), are mainly pollinated by birds or bats. This suggests that stickiness in flowers has mostly evolved as a trait that reduces interactions with insects, (or at least short-tongued insects) with a lesser effect on avian pollinators. Stickiness in petals as a trait to reduce nectar robbing has recently been suggested in other species of Ericaceae (McCarren et al. 2021), but the consequences of such robbing on plant fitness have thus far not been evaluated.

Nectar robbing does not affect plant female fitness

Given the strong impact of nectar robbing on the pollinator –plant mutualism, the effects of nectar robbers can be predicted to be mainly negative for plant fitness. However apparently neutral results are not uncommon and have been found in other systems (Morris 1996; Maloof and Inouye 2000; Arizmendi 2001; Irwin et al. 2015). In the *B. resinosa* study system, these neutral effects on plant fitness could be due to the self-compatible mating system of *B. resinosa*, in conjunction with the presence of viscid threads. One single thread contains all the pollen of one of the anthers, so that, a single visit from a pollinator could move one single viscid thread that contains enough pollen grains to guarantee pollination of a full set of ovules and thus full seed set per flower. The effect of this reproductive assurance trait is further potentiated by the fact that the flowers can last for up to 12 days. A visitation of the flower by any pollinator within this time frame is very likely even under pollinator-limited circumstances. This could potentially eliminate the negative effects on seed sets that result from the deterrence of pollinators, directly by sticky petals, or indirectly by differential effects of stickiness on nectar robbers. In general, self-compatible plants have lower levels of pollen limitation compared with self-incompatible plants (Burd 1994; Larson and Barrett 2000).

While nectar robbing does not seem to have any effect on fruit set, the clear effects of stickiness on primary and secondary nectar robbers and their potential interactions with pollination might still affect outcrossing rates and so affect plant fitness. Moreover, our previous findings suggest additional interactors as potential major agents of selection, including florivores and pollinators that maintain the stickiness dimorphism in *B. resinosa* populations (Chauta in.rev). In the study area, many flowers are attacked by a moth from the Tortricidae family. The larvae enter the flowers and consume all internal structures of the flower (stile, anthers, ovaries), which reduces the reproductive output of these flowers to cero. Stickiness has been demonstrated to reduce attacks by those caterpillars and affect fruit set (Chauta in.rev.). Another possible explanation for the lack of effect of nectar robbing could be that primary nectar robbers function as pollinators, even if as relatively inefficient once. However, in a study on pollination of plants in The Andes, *Diglossa. spp.* were never observed pollinating *B. resinosa* (Rojas-Nossa 2007). These results show that stickiness in *B. resinosa* corolla provides defenses against nectar robbers, which could potentially affect outcrossing rates.

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Chapter 4

Metabolic integration of spectral and chemical cues mediating plant responses to competitors and herbivores

Authorship to be published as Alexander Chautá and André Kessler. Metabolic integration of spectral and chemical cues mediating plant responses to competitors and herbivores.

Abstract

Perception and interpretation of information are fundamental for the survival of any living organism. This information could come in different forms. In plants, information encoded in light and chemical molecules provides information about the presence and nature of competing neighbors and potentially damaging herbivores in the environment. For example, the phytochrome-mediated perception changes in red: far-red light ratios allow plants to decode information about the presence and identity of competitors. Similarly, volatile organic compounds (VOC) encode information about the metabolic status and associated pathogen or herbivore attacks of neighboring plants, which can be a reliable predictor of future attacks for a receiver plant. Both light and VOCs change the regulation of gene expression involved in the growth and production of secondary metabolites. We hypothesize that plants can integrate the information available about competitors and future herbivory to optimize metabolic responses and so negotiate the combined negative fitness effects of multiple stressors. We predict that plant-induced responses to herbivory as well as the VOC-mediated perception of herbivory in the environment are changed by the presence of neighbors. To test this hypothesis, we first manipulate the presence of a neighboring plant by using a far-red light supplementation and herbivory to study their isolated and combined effect on secondary metabolite induction. Second, we exposed plants to supplemental far-red (FR) light as well as to VOCs from control or herbivore-damaged plants to study the effect of the perception of a potentially competitive neighbor on the plant's perception of VOCs emitted by herbivore-attacked neighboring plants. We found strong interactions between the exposure to FR light and damage on the induction of chemicals (volatile and non-volatile). Similarly, but to a lesser extent, the perception of VOCs emitted from neighboring plants was altered by the simultaneous exposure to FR light. These results suggest that plants integrate spectral and chemical environmental cues to change the production of VOC and non-volatile compounds.

Introduction

The ability to perceive, process, and integrate information from the environment is essential for any kind of behavioral or phenotypically plastic response and thus for the fitness of any organism (Chen et al. 2004). Plants are not an exception and, much like animals, have been shown to perceive and process information coded in light (Carvalho et al. 2011), sound (Khait et al. 2019), volatile organic compounds (VOCs) (Heil and Ton 2008), and touch (Mishra, Ratnesh Chandra Bae 2019). Out of these, the perception of light and VOCs have received increased recent attention as they can encode information about the most important antagonistic interactions plants can have with other organisms, competition, and herbivory/pathogen attack, respectively (Karban 2008). Plants perceive light with pigment molecules, such as chlorophylls and phytochromes. While chlorophylls are the key pigments for light capturing and photosynthesis, phytochromes regulate processes such as germination, etiolation, shade avoidance, floral induction, induction of bud dormancy, tuberization, tropic orientations, and proximity perception (Smith 1995; De Wit et al. 2013). Phytochromes are present in two interconvertible forms: P_r and P_{fr}, and the relative cytosolic concentrations of P_r and P_{fr} are determined by environmental light quality (Rockwell et al. 2006). Specifically, a low ratio of Red: Far-Red (R: FR) light transforms phytochrome into its inactive form (P_r), which attenuates the degradation of PIF (phytochrome interacting factors), which, in turn, leads to different physiological changes in the plant. Most importantly for this study, a low R: FR ratio, as reflected off of green leaves, allows plants to perceive potential competitors in their vicinity and invest energy in competition through, for example, accelerated elongation growth (Casal et al. 1987). Accordingly, experiments on tobacco and tomato have demonstrated that this phytochrome-mediated perception of changes of the R: FR light ratio is also associated with a reduced investment into direct resistance to herbivores and the attenuation of induced resistance (Izaguirre et al. 2006; Cortés et al. 2016). Thus, the plants' ability to perceive changes in the R: FR light ratio allows a fine-tuning of the allocation of resources into competition or anti-herbivore defenses. Interestingly, as with resistance-mediating metabolites, the R: FR ratio alone can also affect the production of VOCs (Colquhoun et al. 2013; Kegge et al. 2015; Carvalho et al. 2016), and non-volatile compounds (Tegelberg et al. 2004; Kuo et al. 2015). However, the direction of the effect on individual compounds may vary. Most importantly, a low ratio of R: FR makes plants reduce jasmonic acid (JA) and salicylic acid (SA) mediated gene expression, which, in turn, makes plants more susceptible to damage by pathogens (De Wit et al. 2013) as well as herbivores by affecting the expression of induced resistance traits in response to the attackers. These typical response patterns have been interpreted as an R: FR mediated allocation of resources away from defenses and into competitive ability (Leone et al. 2014).

Similar to the perception of differences in light quality, the ability of plants to produce and perceive volatile organic compounds (VOCs) seems to play an essential role in coping with multiple environmental challenges. VOCs are crucial in mediating plant direct and indirect resistance against herbivores (Dudareva et al. 2013). After damage by herbivores plants emit increased and attacker-specific blooms of VOCs, often called herbivory-induce plant volatiles (HIPV)(Becker et al. 2015). These HIPVs are often repellent to foraging herbivores but can also function as effective cues that attract natural enemies of herbivores, such as predators and parasitoids (information-mediated indirect defenses) (Dicke and Baldwin 2010; Becker et al. 2015). Moreover, HIPVs can also be perceived by other plants, which respond by priming or directly inducing increased production of defense-related secondary metabolites and so increased resistance in anticipation of oncoming herbivores (Karban et al. 2011; Okada et al. 2015; Morrell and Kessler 2017; Kalske et al. 2019; Karban 2021). The mechanisms of plant VOC perception are debated to this date but include altered membrane potential induction, the potential existence of specific receptors, and the transformation of VOCs into direct defensive compounds by the receiver plants (Erb 2018). However, very much like shifts in R: FR light ratios encode potential competition with neighbors, HIPVs provide a reliable cue for the probability of future herbivory. The fact, that plants have these different abilities to adaptively respond to changed light quality and HIPVs from neighbors, in turn, raises the question of how plants integrate these two different types of information to optimize responses to two of the most fitness-impacting environmental factors, competition, and herbivory.

Here we address this question in a community and chemical ecology model system, the Tall Goldenrod, *Solidago altissima. Solidago altissima* dominates early succession habitats in open environments in northeastern North America (Etterson et al. 2008; Howard et al. 2018). This species grows in dense patches where it competes for light with a diverse Astereacea-dominated plant community. Additionally, this plant is attacked by a large diversity of insect herbivores (Maddox and Root 1987, 1990). Most importantly, however, plant community composition (Carson and Root 2000), as well as population genetic composition (Bode and Kessler 2012; Uesugi and Kessler 2013), are driven by a strong interaction between competition and insect herbivory, which determines the ecological circumstances in which interactions of dominant species *S. altissima* with other organism is played out. Moreover, previous studies have demonstrated that *S. altissima* plants strongly respond to HIPVs from neighboring plants by priming and directly inducing changes in secondary metabolism and resistance (Morrell and Kessler 2017). Moreover, HIPV-mediated plant-to-plant information transfer affects herbivore distribution (Rubin et al 2015) and is under herbivory-mediated natural selection (Kalske et al 2019). This raises the question, what are the mechanisms that allow plants to minimize the combined, often synergistic impact of antagonistic biotic factors. The hypothesis that we are addressing here is that plants can integrate the information available on future herbivory and competition to induce metabolic changes that minimize the negative fitness effects of multiple interacting antagonists. Here we test two major predictions associated with this hypothesis: A) Secondary metabolite responses to herbivory should be altered in the presence of a neighbor (i.e. perception of lower R: FR ratio). B) Perception of oncoming herbivory (i.e. HIPVs from damaged neighbors) should be altered by the presence of a potentially competitive neighbor (i.e. perception of lower R: FR ratio). This hypothesis seems particularly relevant in the study system we chose for this project, the tall goldenrod *S. altissima.* In this species, herbivory can be the major factor mediating competition with neighbors (Carson and Root 2000, Uesigi and Kessler 2013), so that a fine-tuned , integrated response to the combined perception of competitors and herbivores can be predicted to disproportionately affect plant fitness . Here we use factorial manipulative experiments to address the above-mentioned hypothesis and further our understanding of how plants integrate two different sources of biotic environmental information (light and HIPVs).

Materials and Methods

Plant material

Seeds of *S.altissima* were bulk-collected in winter 2020 from plants around Beebe Lake, Ithaca, NY, and then stored in a freezer at -20°C. After a month, the seeds were put into LM1 germination mix soil (Lambert) for germination at a Cornell University's greenhouse with a photoperiod of 12:12 light: dark. Once the plants had germinated, they were repotted into individual clear plastic cups of 500mL capacity, and an initial measurement of the length of the plant and the number of leaves was taken. Therefore, all neighboring plants in this experiment were assumed to be unrelated to each other. All plants were grown under high-pressure Sodium lamps that produce 200 μmol/m^2/sec of white light in a photoperiod day-night 12:12. In addition, half of the plants were under supplemental Far-Red (FR) light, using an FR lamp (Forever Green Indoors, 730 nm) of 114 cm with 32 LED bulbs. The FR lamps were covered with a blue filter (Roscolux, Supergel, Cinegel no. 83 Medium Blue) to remove residual red light following the protocol of Fernández-Milmanda *et al.* (2020). The lamp was located at 15 cm to the side of the plant and 10 cm from the ground to simulate the angle of light reflected off of a neighboring plant (Fig. 4.1A). After one week, the second measurement of height and number of leaves was recorded to assess the effect of increased ratios of FR light on plant growth. After these measurements, two larvae of

Spodoptera frugiperda in their third instar were added to each of 10 plants in each light treatment (FR and control), completing four groups of plants: Control, Damage, FR, and FR + Damage (Fig. 4.1B). After another four days with the larvae actively feeding. At this point, 10 plants in the damage treatment and 10 plants in the control treatment were used as emitter plants in a plant VOC-exposure experiment. Their VOC emissions were pulled into receiver plant chambers that included either control plants under normal light conditions or plants supplemented with FR light (Fig 4.1C). The chambers of both the emitter and receiver plants were connected through a hose of 0.7 cm in diameter made of clear vinyl, and the chamber of the receiver was connected to an active air sampling vacuum pump (IONTIK) pulling air at about 450 ml/min. The pumps generate a constant flow of air from the emitter to the receiver plants (Fig. 4.1C). The pumps were exchanged with recharged ones twice a day, to ensure that there would be 22 hours of flow per day. After four days of VOC exposure, we collected VOCs using adsorbent traps and leaf material to analyze non-volatile metabolites. Volatile samples of each plant were taken by enclosing the plant into 500 mL polyethylene cups that were connected to an ORBO-32 charcoal adsorbent tube (Supelco®). The air was pulled through the charcoal traps using an active air sampling vacuum pump (IONTIK) pulling air at about 450 ml/min. Additionally, leaf samples were collected and put immediately on liquid nitrogen and later stored at -80°C for posterior analysis. To understand if the chemical response to damage is affected by the presence of a neighbor, we compared the volatile and non-volatile chemical profiles of the emitter plants (Control, Damage, FR, and FR+Damage). To understand if the perception of volatiles is affected by FR exposure we compared the volatile and non-volatile, secondary metabolites produced by the receiver plants.

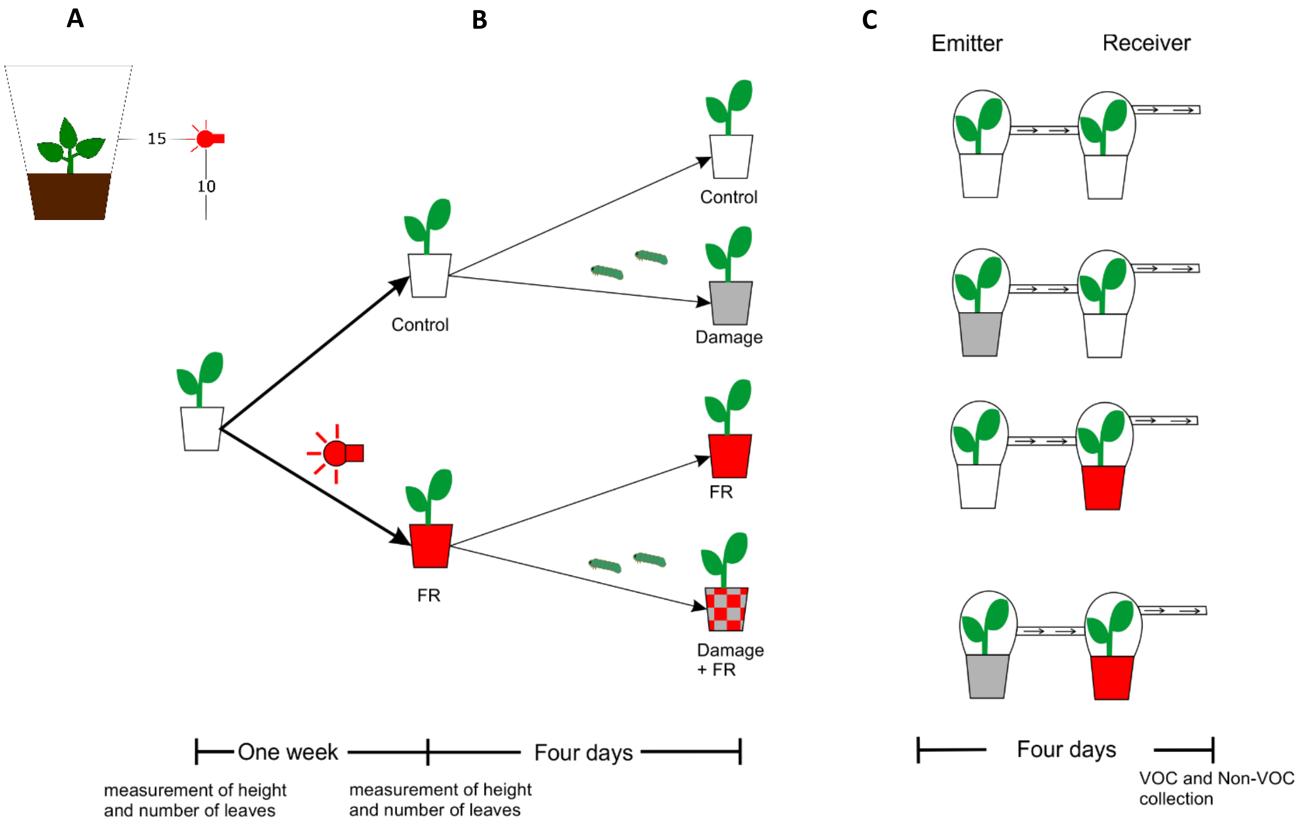


Figure 4.1. A. Arrangement of plants and FR lamps for the experiment. **B.** Scheme showing the sequence of treatments of plants of *Solidago altissima*. Plants were divided into two groups; one was exposed to far-red (FR) light and the other was kept as a control. Then half of the plants in each treatment were damaged for four days by two *Spodoptera fruguiperda* larva in L2. **C.** Plants in the FR light and control treatments were set up to receive volatile organic compounds (VOC) from plants that were damaged by *S. fruguiperda*, for four days or from control plants that received no damage.

Secondary metabolite analysis

Before elution, each of the ORBO-32 charcoal traps was spiked with 5µL of tetraline (90 ng/mL) as an internal standard. The charcoal traps were washed with 400µL of dichloromethane, which was then injected in a Varian CP-3800 gas chromatograph (GC) coupled with a Saturn 2200 mass spectrometer (MS) and equipped with a CP-8400 autosampler. The GC-MS was fit with a DB-WAX column, (Agilent, J&W Scientific) of 60 m × 0.25 mm id capillary column coated with polyethyleneglycol (0.25 mm film thickness). The temperature program began with an injection temperature of 225°C, heated from 45_C to 130°C at 10°C/minute, then from 130°C to 180 at 5°C/min, and finally from 180°to 250°C at 20°C/minute with a 5 min hold at 230 and 250°C. The samples were standardized by expressing signal intensity relative to the area of the internal standard.

For the high-performance liquid chromatography (HPLC) analysis of non-volatile compounds, leaf samples were homogenized and extracted in 1 mL of 90% methanol using a FastPrep® tissue homogenizer (MP Biomedicals®) at 6 m/s for 90 s using 0.9-g grinding beads (Zirconia/Silica 2.3 mm, Biospec®). The samples were then centrifuged at 4 °C for 15 min at 14,000 rpm and analyzed 15 µL of the by HPLC on an Agilent® 1100 series HPLC. Using 99.9% acetonitrile and 0.25% H_3PO_4 as mobile phase. The elution system consisted of aqueous 0.25% H3PO4 and acetonitrile (ACN) which were pumped through a Gemini C18 reverse-phase column (3 µm, 150 × 4.6 mm, Phenomenex, Torrance, CA, USA) at a rate of 0.7 mL/min with increasing concentrations of ACN: 0–5 min, 0–20% ACN; 5–35 min, 20–95% ACN; and 35–45 min, 95% ACN. The area of each peak was standardized by the mass of the leaf tissue extracted. The compound class was determined based on the UV pattern.

Statistical analysis

The differences in growth between FR and control plants were analyzed using a Students' t-test. The overall composition of volatile and non-volatile plant secondary metabolites was inspected using nonmetric multidimensional scaling (Bray–Curtis distance matrix; metaMDS in *vegan* package) and tested for the effects of the FR light exposure and herbivory on the composition with a PERMANOVA with 999 permutations using the adonis2 function in the vegan package using the trials as strata. For the emitters, we used the exposure to FR and damage as independent factors, and the relative abundance of compounds as dependent factors. For the receivers, we used the exposure to FR and the damage on the emitter plant as independent factors, and the relative abundance of compounds as dependent factors. If PERMANOVAs had shown significant results, a *post hoc* test was run using the function *pairwise.adonis2* from the library *pairwise.adonis* (Martinez Arbizu 2020). We adjusted the P values for the multiple corrections using the false discovery rate (FDR) adjustment (*p. adjust* in package *stats)*. Additionally, individual ANOVAs were run for each volatile and non-volatile compound from emitter and receiver plants. Those that showed variation with treatments were included in a heatmap analysis for easier visualization of the complex differences. All statistical analyses were performed using the R program (R Team Core 2021).

Results

Effect of FR light on plant growth

Exposure to supplemental FR light resulted an increased stem elongation relative to plants under normal light conditions (t = -10.264, df = 63, p-value < 0.001, Fig 4.2A), however there was no effect on then number of new leaves that grew between measurements (t = -0.93743, df = 63, p-value = 0.3521, Fig.4.2B).

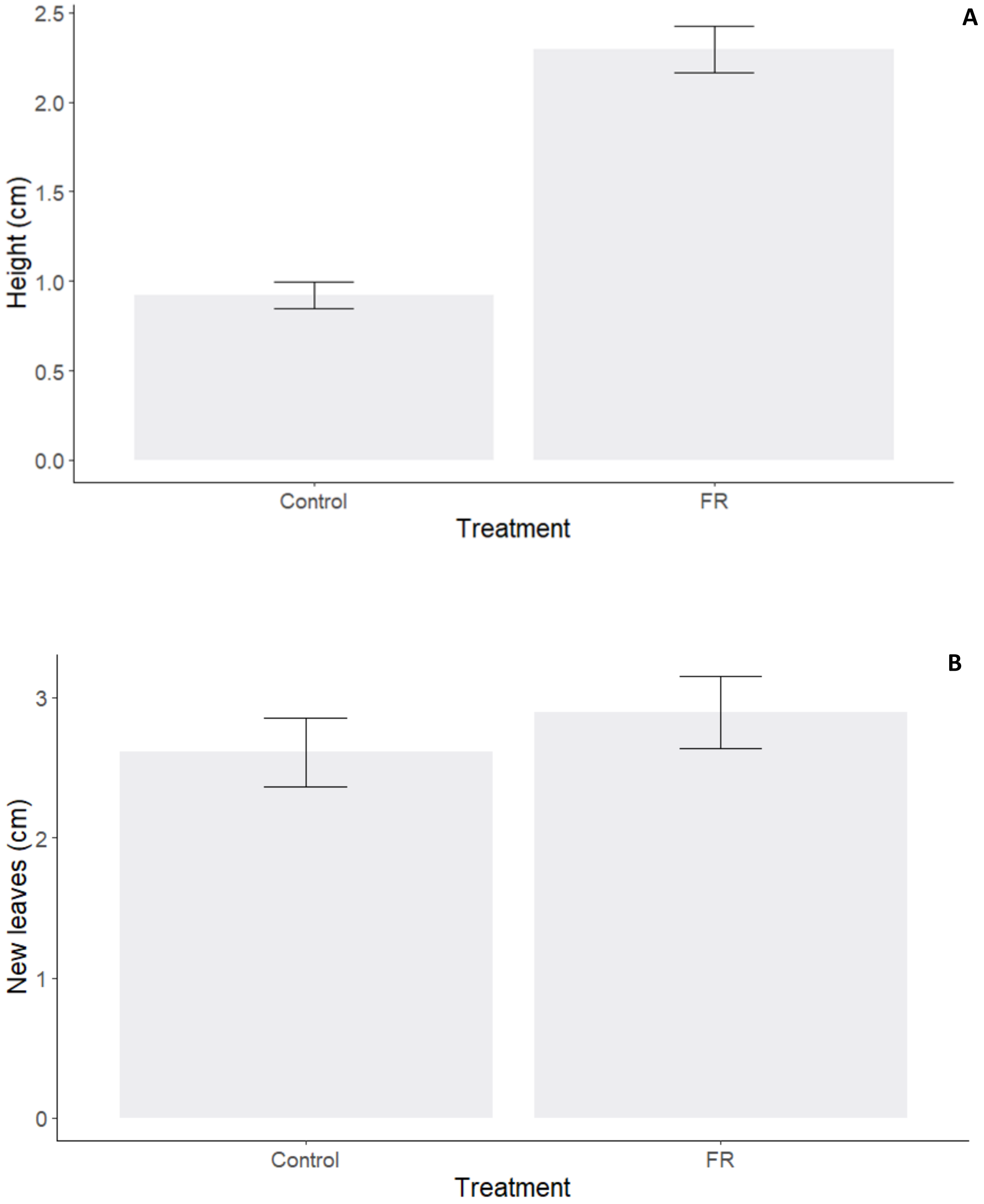


Figure 4.2. A. Plant growth responses to far-red (FR) light exposure. Mean (±SEM) stem height and **B.** Mean (±SEM) number of new leaves produced by *Solidago altissima* plants growing under regular light conditions supplemented with FR light (reduced red: far-red ratio) or under regular (control) light conditions over one week.

Effect of herbivory and FR light on plant chemistry

Both FR exposure and herbivore damage influenced plant VOC production (PERMANOVA, $F_{1,36}$=9.5016, p=0.001 and $F_{1,36}$= 4.3728, p=0.007 respectively) and we identified a strong interaction between both factors (FR x herbivore damage, $F_{1,36}$= 7.2251, p=0.001).

The *Post hoc* analyses revealed differences between plant headspace VOC emissions in response to all the experimental treatments except for the comparison between the emissions from damaged plants, and those from double-exposed FR+Damage plants (Table 4.1). These effects were also reflected illustrated in an NMDS analysis (Fig. 4.3A, stress value= 0.1150099).

Table 4.1. *Post hoc* comparison of the headspace VOC emissions from S*olidago altissima* plants that had been grown either under control light (Control), were damaged by two second-instar larvae of *Spodoptera frugiperda* (Damage), were exposed to supplemented far-red light (FR), or were exposed to both supplemented FR light and damage (FR+Damage) simultaneously. *Represent statistical differences (p<0.05).

Treatment	**Control**	**Damage**	**FR**
Damage	$F_{1,18}$= 3.872, p=0.001 *		
FR	$F_{1,18}$= 19.116, p=0.001 *	$F_{1,18}$= 7.847, p=0.001 *	
FR+Damage	$F_{1,18}$= 5.7041 p=0.001 *	$F_{1,18}$= 0.861, p= 0.283	$F_{1,18}$= 7.7928, p=0.001 *

The separate analysis of individual compounds found 30 volatile secondary metabolites whose production varied with the treatments and was mostly increased in response to damage or the combination of FR exposure and damage (Fig. 4.5A).

Overall nonvolatile compound compositions were also affected by FR exposure (PERMANOVA, $F_{1,36}$=3.2915, p= 0.011) but only marginally by damage alone ($F_{1,36}$=2.0827, p=0.060). However, we observed a strong interaction between both factors (FR x herbivore damage, $F_{1,36}$=5.4194, p=0.001) on the production of non-volatile secondary metabolites.

The *Post hoc* analyses revealed differences in the non-volatile compound production that was induced in response to all applied treatments except for the comparison between damage vs FR+Damage and Control vs FR+Damage (Table 4.2), illustrated in the NMDS analysis (Fig. 4.3B, stress value= 0.2587829).

Table 4.2. *Post hoc* comparison of the non-volatile secondary metabolite production of *Solidago altissima* plants that had been grown either under control light (Control) conditions, were damaged by second instar larvae of *Spodoptera frugiperda* (Damage), were exposed to supplemented far-red light (RF), or experienced the simultaneous exposure to both supplemented FR light and herbivore damage (FR+Damage). *Represent statistical differences (p<0.05).

Treatment	**Control**	**Damage**	**FR**
Damage	$F_{1,18}$= 2.5411, p=0.03 *		
FR	$F_{1,18}$= 7.8519, p=0.003 *	$F_{1,18}$= 3.7581, p=0.001 *	
FR+Damage	$F_{1,18}$= 1.33, p=0.153	$F_{1,18}$= 0.887, p= 0.461	$F_{1,18}$= 4.7507, p=0.002 *

on the separate analysis of individual compounds identified 13 non-volatile secondary metabolites that show a pronounced increase in response to two treatments (Damage and FR) but whose production tended to be lower in the combined FR+Damage treatment (Fig. 4.5B). Of those compounds, seven are diterpenoids, two coumaric acid derivatives, one flavonoid, one chlorogenic acid derivative, and one currently unknown compound.

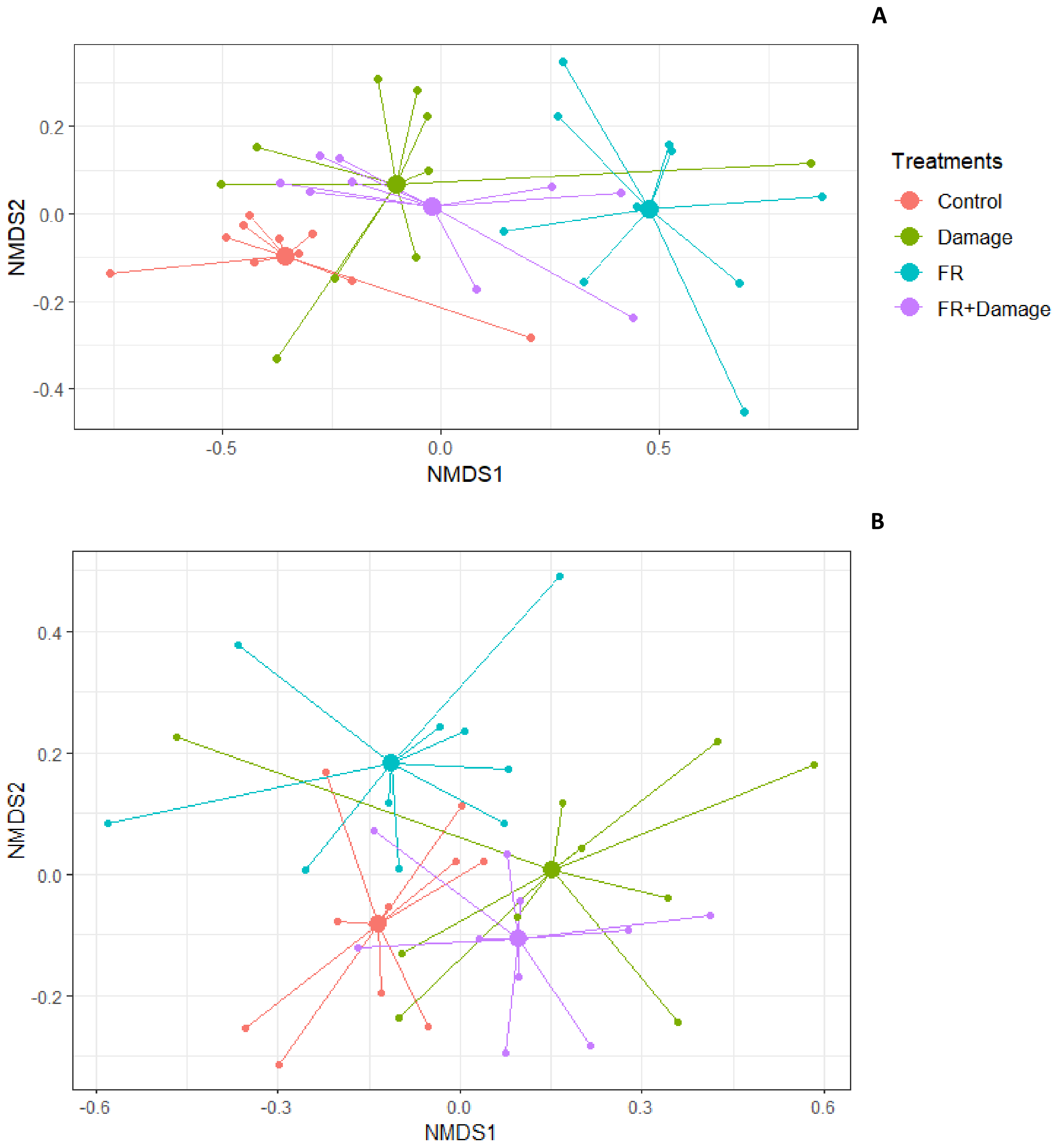


Figure 4.3. Plant secondary metabolite production in response to supplemented far-red (FR) light exposure and herbivore damage. Non-metric multidimensional scaling (NMDS) of **A)** volatile organic compound emissions (stress value=0.115) and **B** non-volatile secondary metabolite production (stress value=0.163) of *Solidago altissima* plants, growing under reduced

red: far-red light ratios (FR), with damage by larvae of *Spodoptera frugiperda* (Damage), the combination of FR and damage (FR+Damage) or under control light (control) conditions.

Effect of FR on the perception of volatiles

The overall composition of VOCs emitted from plants that were exposed to VOCs from neighboring plants did not change with exposure to increased FR light ratios ($F_{1,36}$= 1.444, p=0.095) or with the exposure to VOCs from damaged plants ($F_{1,36}$= 1.459, p=0.106), neither did we observe an interaction between both factors ($F_{1,36}$= 1.543, p=0.086. Fig. 4.4A). However, emissions of five individual VOCs varied with the treatments. Four of those VOCs were emitted in higher amounts from plants that were exposed to increased FR light and, at the same time, received VOCs from control plants. However, these same compounds showed lower emission rates when simultaneously exposed to increased FR light and VOCs from damaged neighbors, indicating an integration of the two types of environmental information (Fig. 4.6A). The fifth compound was emitted in higher amounts from plants exposed to FR light while also receiving VOCs from damaged neighbors (Fig. 4.6A).

Like the VOC responses, overall non-volatile compound composition from plants that were exposed to increased FR radiation or were exposed to VOCs from herbivore-damage plants did not change ($F_{1,36}$= 1.50148, p=0.119, $F_{1,36}$= 0.85814, p=0.497 respectively) relative to that of control plants. We did also not observe differential secondary metabolite production resulting from the interaction between FR and Damage ($F_{1,36}$= 0.96695, p=0.380, Fig 4.4B). However, the separate analysis of individual compounds found nine non-volatile secondary metabolites whose production varied with the treatments (Fig. 4.6B).

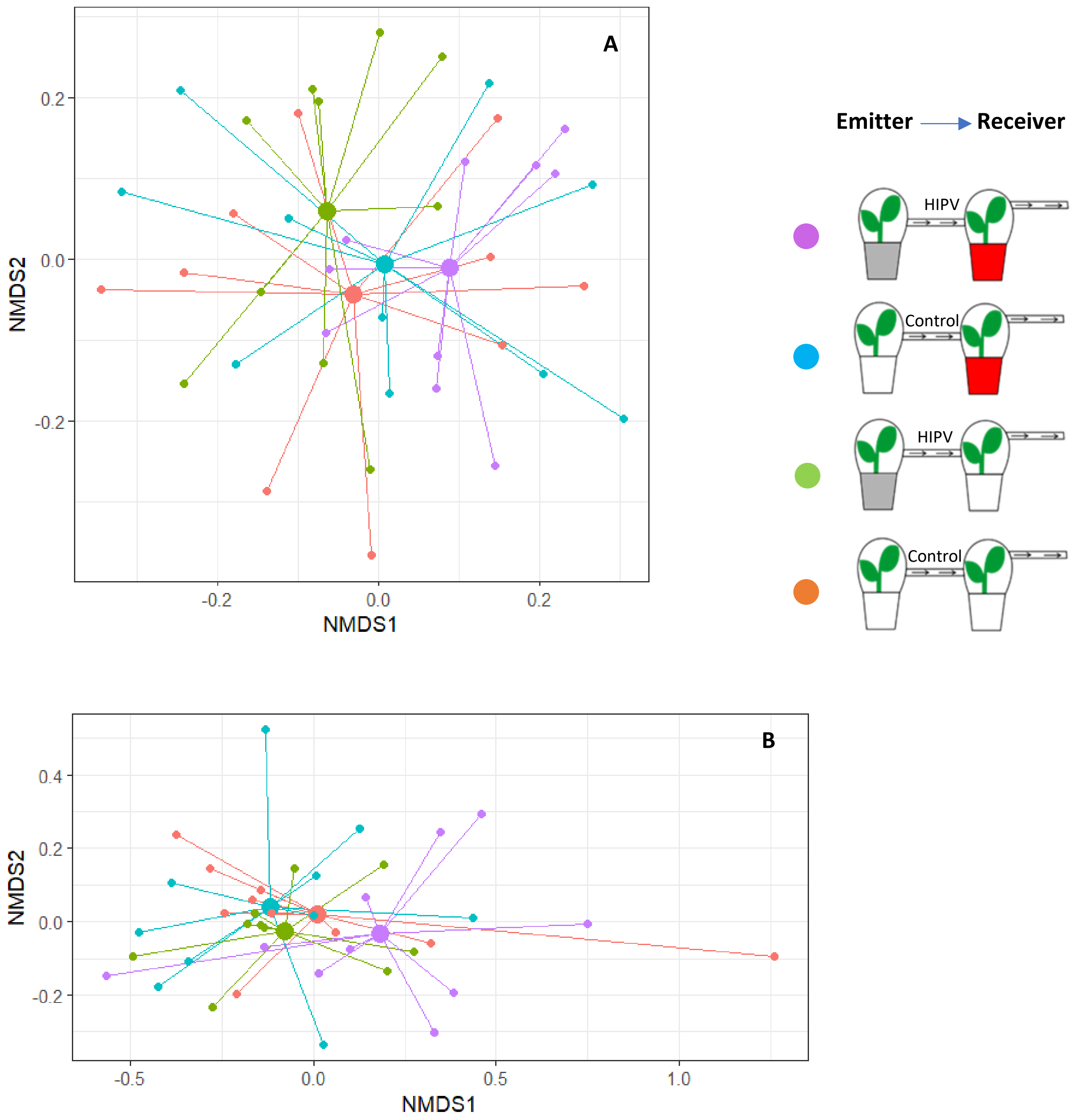
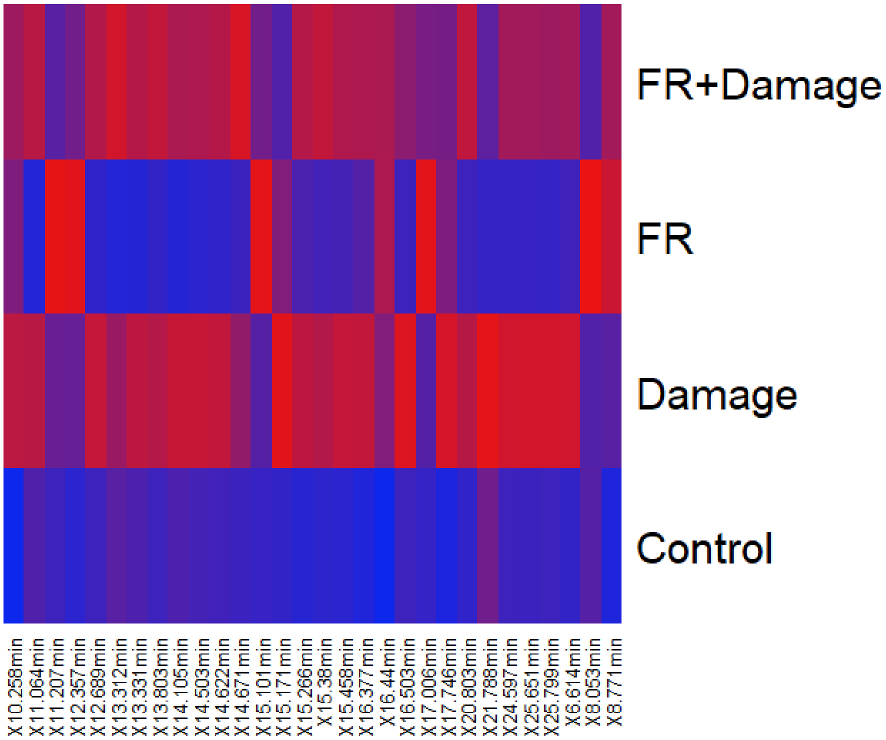


Figure 4.4. Plant secondary metabolite production of *Solidago altissima* plants under supplemented far-red light (Red pots) or normal light (white pots), that were exposed to volatiles from control plants or herbivore-induced plant volatiles (HIPVs) from plants that have been damaged by two larvae of *Spodoptera furgiperda* (grey pots). Non-metric multidimensional

scaling (NMDS) of **A)** volatile organic compound emissions (stress value=0.08728402) and **B** non-volatile secondary metabolite production (stress value=0.2796113).

A


FR+Damage
FR
Damage
Control
X10.258min
X11.064min
X11.207min
X12.357min
X12.689min
X13.312min
X13.331min
X13.803min
X14.105min
X14.503min
X14.622min
X14.671min
X15.101min
X15.171min
X15.266min
X15.38min
X15.458min
X16.377min
X16.44min
X16.503min
X17.006min
X17.746min
X20.803min
X21.788min
X24.597min
X25.651min
X25.799min
X6.614min
X8.053min
X8.771min

B

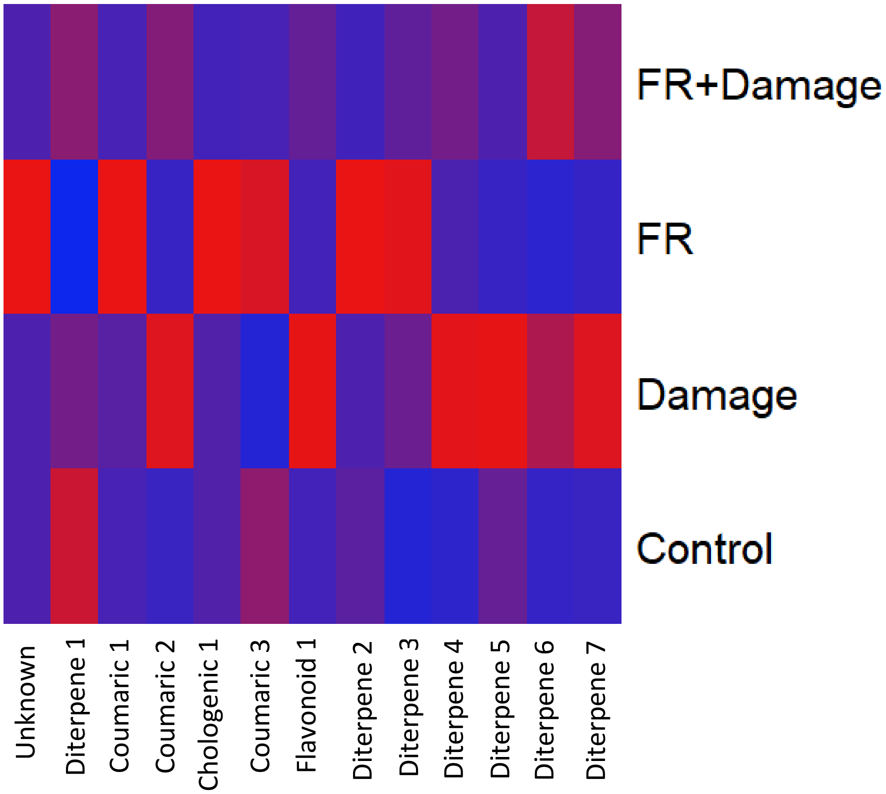

FR+Damage
FR
Damage
Control
Unknown
Diterpene 1
Coumaric 1
Coumaric 2
Chologenic 1
Coumaric 3
Flavonoid 1
Diterpene 2
Diterpene 3
Diterpene 4
Diterpene 5
Diterpene 6
Diterpene 7

Figure 4.5. Differential induction patterns of individual secondary metabolites in response to supplemental far-red (FR) light exposure and herbivory (damage). Heat map of (A) the emission of volatile organic compounds (VOCs) and (B) the production of non-volatile compounds whose production is significantly varying with treatment ($p<0.05$). The different treatments include untreated controls, plants exposed to increased FR radiation (FR), plants damaged by *Spodoptera frugiperda* caterpillars (Damage), and plants that received both treatments (FR+Damage). The color represents the amount of each compound whereby blue represents low amounts, while red is high amounts relative to the control.

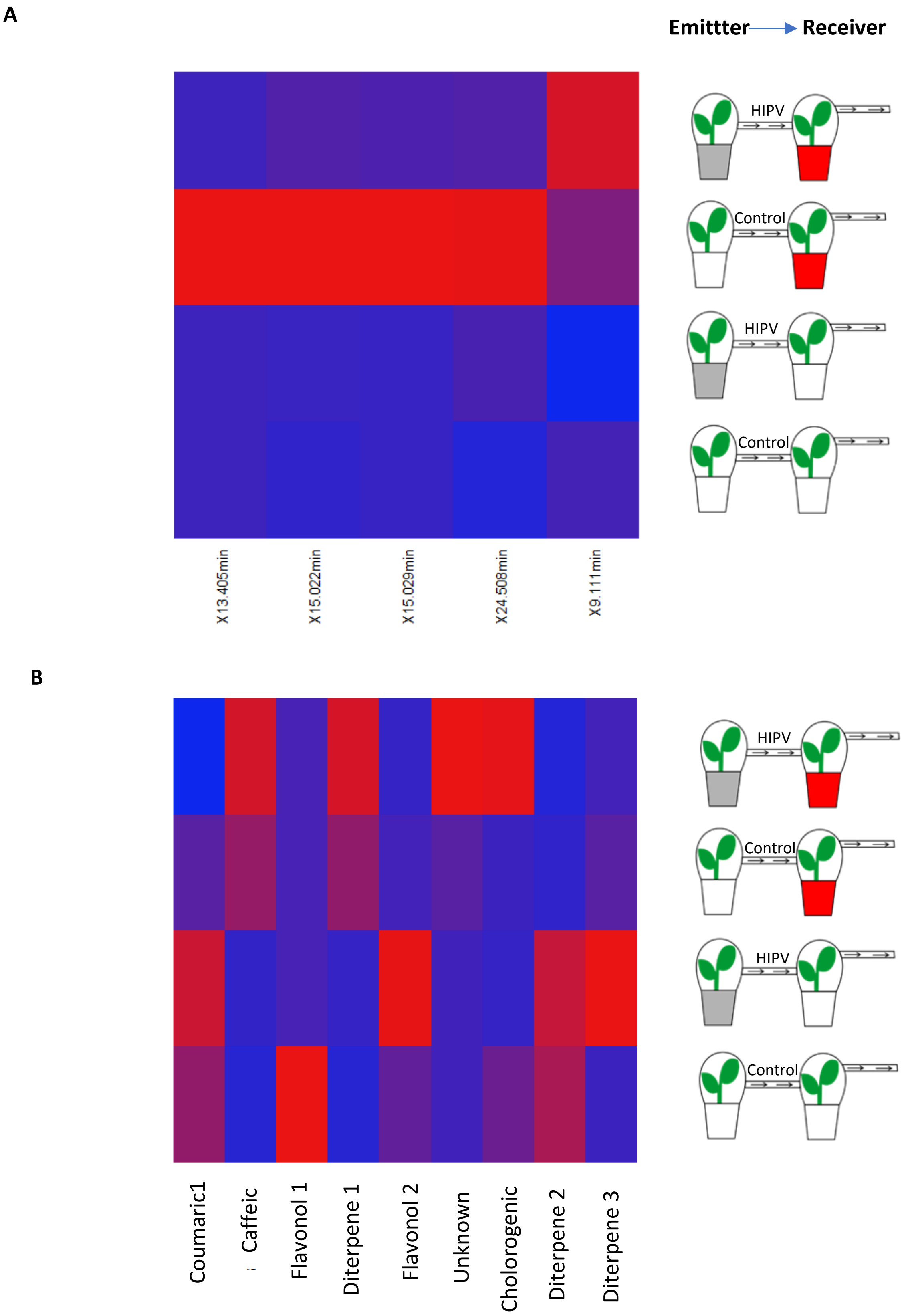

A
Emittter
Receiver
HIPV
Control
HIPV
Control
X13.405min
X15.022min
X15.029min
X24.508min
X9.111min
B
HIPV
Control
HIPV
Control
Coumaric1
Caffeic
Flavonol 1
Diterpene 1
Flavonol 2
Unknown
Chologenic
Diterpene 2
Diterpene 3

Figure 4.6. Differential induction patterns of individual secondary metabolites in response to the exposure to herbivory-induce plant volatiles (HIPVs). Heat map of (A) the emission of VOCs and (B) the production of non-volatile compounds whose production is significantly varying with treatment ($p<0.05$). The different treatments include untreated controls, plants exposed to increased FR radiation (FR), plants that were exposed to HIPVs from neighboring plants damaged by *Spodoptera frugiperda* caterpillars (HIPV), and plants that received both treatments (FR+HIPV). The colors represent the amount of each compound, whereby blue indicates low amounts, while red color indicates high amounts relative to the control treatment.

Discussion

Plants can perceive neighbors by the shift in the R: FR ratio of light reflected off of green leaves and are commonly observed to respond with accelerated stem elongation (Demotes-Mainard et al. 2016). In confirmation of these earlier findings, we found *S. altissima* plants responding in a very similar way. The exposure of the plants to increased FR light radiation increased stem length, but not the number of leaves. This standard growth response to perceived potential competitors is commonly interpreted as an adjustment of the plant's metabolism and an apparent allocation of resources into competitive ability and away from defensive functions (Izaguirre et al. 2006; Leone et al. 2014). A further indication of such a metabolic reconfiguration was apparent in the altered volatile and non-volatile secondary metabolite production associated with the increased exposure to FR light. *Solidago altissima* plants exposed to supplemented FR light, and herbivory change the production of secondary metabolites (volatiles and non-volatiles) with a strong interaction between both factors. Similarly, but to a somewhat smaller extent, the ability of plants to perceive volatiles coming from neighboring plants (with and without damage) was affected by the exposure to FR light, suggesting integration of spectral and chemical cues by the plant.

Growth under FR light

In shade-intolerant plants, a low ratio of R: FR light induces differential growth such as stem elongation (Fankhauser and Batschauer 2016) that can provide a competitive advantage over neighbors in the natural habitats (Demotes-Mainard et al. 2016). The stem elongation induced by higher FR light proportions usually results from an increase in the internode distance rather than an increase in the number of internodes (Demotes-Mainard et al. 2016), because the elongation effect is caused by gibberellin A1 and Indole-3-acetic acid (IAA)-mediated cell expansion (Kurepin et al. 2007; Pierik et al. 2014). This, in turn, explains why we did not also find a change in the number of leaves in FR-supplemented *S. altissima* plants. Similar plant-endogenous signaling mechanisms are thought to also mediate the correlated changes in plant secondary metabolism and the changes in the inducibility of metabolic responses to other environmental cues and stressors, such as herbivory (Izaguirre et al. 2006). Thus, the functional question for why plants induce changes to competition-indicating light quality has to be answered on two levels. On one hand, we need to explain why secondary metabolism changes in response to different light quality in the first place (i.e., the potential benefit of altered constitutive defenses). And, on the other hand, one has to probe the potential effects of light-quality-mediated changes on the perception of other stressors, such as herbivory (i.e., integration of environmental information from different sources).

Effect of increased FR on constitutive and herbivory-induced secondary metabolism

Effects of FR light on secondary metabolite production, specifically volatile compounds, have been shown in *Petunia × hybrida* (Colquhoun et al. 2013), *Hordeum vulgare* (Kegge et al. 2015), *Ocimum basilicum* (Carvalho et al. 2016), and now *S. altissima*. The previous studies suggest the involvement of a wider range of phytohormones that had been found important for the growth responses. For example, a low R: FR ratio causes downregulation in the jasmonic acid (JA) pathway in shade-intolerant plants (Leone et al. 2014; Fernández-Milmanda et al. 2020). This pathway is crucial in the induced production of defensive compounds in plants, but commonly reduces plant growth (Cipollini and Lieurance 2012). In *Arabidopsis thaliana*, JA is repressed by low R: FR light ratios (Leone et al. 2014) suggesting a priority of growth over defenses. Interestingly, the increased expression of IAA signaling, induced by a low R: FR ratio, has long been known as an inhibitor of JA responses and may explain the differential allocation of resources into cell elongation and away from secondary metabolism (Mason and Mullet 1990; Thornburg and Li 1991; Mason et al. 1992; Dewald et al. 1994). Moreover, the inhibition of wound- or herbivory-induced JA signaling will also significantly impair the herbivory-mediated induction of defense-related secondary metabolites (Baldwin et al. 1997). This would certainly explain the aforementioned findings on the FR-induced allocation into growth away from constitutive and induced secondary metabolite production and resistance in the tobacco *Nicotiana sylvestris* and the tomato *Solanum lycopersicon* (Izaguirre et al. 2006; Cortés et al. 2016). From a functional perspective, this kind of response is likely adaptive in plant systems where competition with neighbors is substantially more impacting on plant fitness than herbivory. In systems like *S. altissima* where herbivory can be the major factor mediating competition with neighbors (Carson and Root 2000, Uesigi and Kessler 2013), more nuanced and integrated response to the combined perception of competitors and herbivores may suit the plants better. While this project focused on the evidence for such an integration of different types of information, it goes beyond the scope of this paper to investigate the actual resistance and plant fitness effects.

However, in the light of both, the wider functional hypothesis as well as in the light of the objective of this study, there are several remarkable induction patterns in *S. altissima's* response to FR light and herbivory. Previous studies have been shown that FR light and damage affect the production of VOCs in plants (Colquhoun et al. 2013; Becker et al. 2015; Kegge et al. 2015). When *S. altissima* plants are exposed to both herbivore damage and FR light, the chemical profile becomes more like one of the plants with damage. This suggests that in the case of *S. altissima*, secondary metabolism and its induction by herbivores are not suppressed by FR light. More importantly, the fact, that the combination of FR light supplementation (i.e. perception of a potential competitor) and herbivory induced different volatile and non-volatile secondary metabolite profiles is strong support for an information integration hypothesis. Interestingly, compounds that were mostly up-regulated by FR light in *S. altissima* plants were the ones that are down-regulated with damage or the combined exposure to FR light and herbivory and vice versa (Fig. 4.5A). Non-volatile compounds have been observed to change with increased FR light exposure in other study systems (Tegelberg et al. 2004; Kuo et al. 2015). Similarly, to VOCs, the changes in non-volatiles could be related to changes in the JA pathway. Interestingly, some of the compounds up-regulated by FR light in *S. altissima* are diterpenes (Diterpene 2 and 3, Fig. 4.5B), which are known for having functions as antifeedants and growth inhibitors for *Solidago* herbivores (Cooper-Driver & Le Quesne 1987, Uesugi and Kessler 2016). While previous studies have found downregulation of defenses in response to the exposure to FR light (Izaguirre et al. 2006), this up-regulation of defense metabolites in *S. altissima* indicates a differently regulated response to competition and herbivory. In conclusion, the interaction between FR light and herbivore damage in the volatile and non-volatile chemical profile, suggests that *S. altissima* can integrate both signals (FR light and herbivory) and adjust its chemistry accordingly.

Effect of FR light exposure on the perception of HIPVs from neighbors

Our second prediction in the information integration hypothesis went one step further and suggested that if plants can integrate the information of a perceived neighbor with the information provided by an actively feeding herbivore, plants may also be able to integrate the perceived neighbor with cues that indicate future herbivory (i.e. HIPVs emitted from damage neighboring plants). Overall *S. altissima* secondary metabolite profiles did not differ much in response to the exposure to VOCs from control plants or plants exposed to FR light (Fig. 4.4 A and B). This is not necessarily surprising as exposure to VOCs has rarely been found to induce VOC emission without additional damage to the leaf tissue.

Similarly, only a minor proportion of the metabolome is usually directly induced by exposure to VOCs. For example, in *S. altissima* only about 19 compounds of the non-volatile fraction of the recorded secondary metabolites were directly inducible by VOCs from neighboring plants without additional herbivore damage (Morrell and Kessler 2017). However, here we also found several individual compounds induced by the simple exposure of the plant to neighbor VOCs. Specifically, plants under-supplemented FR light-receiving VOCs from an undamaged control plant increase the production of four VOCs dramatically (Fig.4.6A). This suggests that FR light makes plants more susceptible to VOCs from neighbors. Recent studies have suggested that FR light is used by *Arabidopsis thaliana* as a signal for kin recognition, that mediates interactions among kin neighbors, reducing competition for resources (Crepy and Casal 2015). The FR-induced VOC emission as well as the FR-mediated differential perception of VOCs can provide an alternative and more specific mechanism of kin recognition. In *S. altissima*, in which one individual can be surrounded by several clonal ramets, the identification of the neighbor would be beneficial by avoiding investment into resources for competition against itself.

From the nine non-volatile compounds that were affected by the treatments, the greatest increment was evident in plants that received VOCs from plants with damage (HIPVs, Fig.4.6B), confirming earlier studies that *S.altissima* can detect HIPVs (Morrell and Kessler 2017, Kalske et al 2019). However, the compounds that are up-regulated are not the same in the different exposure treatments. On one hand, control plants exposed to HIPVs increase the production of one coumaric acid derivative (coumaric1), one flavonoid (flavanol 2), and two diterpene acids (diterpenes 2 and 3); on the other hand, plants under increased FR exposure and simultaneously exposed to HIPVs increased the production of chlorogenic acid derivative (chlorogenic), one diterpene acid (diterpene1) and caffeic acid derivative (Caffeic) and an unknown compound. This ultimately indicates that plants exposed to light reflected off of potentially competitive neighbors interpreted the information encoded in HIPVs from herbivore-attacked neighbors differently from plants that stand isolated without neighbors. More generally, our data suggest that VOCs can provide information about the presence as well as about the identity and herbivory status of their neighbors. Concerning our initial hypothesis, these data also suggest that *S. altissima* plants integrate the information encoded in VOCs and the light quality to induce changes in their metabolism.

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