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EAVESDROPPING PLANTS: PLANTS PERCEIVE AND RESPOND TO INSECT ODORS BY PRIMING THEIR ANTI-HERBIVORE DEFENSES

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ABSTRACT

Insect feeding damage is known to induce plant defenses. More recent discoveries have found that plants can also perceive environmental cues associated with the presence of insect herbivores, allowing them to prepare their defenses for future attack. For example, plants can detect insect footsteps or oviposition, and some even use olfactory cues to sense the presence of nearby herbivores. Several studies have found that undamaged plants eavesdrop on volatiles emitted by their insect-damaged neighbors and respond by enhancing their own anti-herbivore defenses. In my dissertation research, I demonstrate for the first time that plants can also perceive and respond to olfactory cues emitted directly by insect herbivores. My findings indicate that tall goldenrod (*Solidago altissima*) plants exhibit enhanced defense responses following exposure to the putative sex attractant of a specialist herbivore, the goldenrod gall fly (*Eurosta solidaginis*).

In field and laboratory experiments, goldenrod plants previously exposed to the male fly emission suffered significantly less herbivore damage than unexposed controls. Moreover, goldenrod plants exposed to the fly odor induced higher amounts of the key defense signaling hormone jasmonic acid and emitted greater quantities of defense-related volatile compounds following herbivore damage. I also found that exposure to the *E. solidaginis* emission does not directly deter subsequent insect feeding as exposing other plant species to the emission did not reduce herbivore feeding damage.

Because little is known about how plants perceive olfactory cues and the specificity of plant olfaction, I also investigated *S. altissima* responses to the individual chemical constituents of the *E. solidaginis* emission blend. I found that goldenrod plants

exposed to the most abundant compound in the blend, (7*S*,5*S*)-7-methyl-1,6-dioxaspiro[4.5]decane, received less herbivore damage and induced higher quantities of jasmonic acid than control plants, a similar result to plants exposed to the complete blend.

Male *E. solidaginis* flies emit very large quantities of the putative sex attractant (~70 ± 20 μg over 24 h), despite providing a cue for eavesdropping *S. altissima* plants. To determine whether female *E. solidaginis* may select for this trait and whether emission production is correlated with *E. solidaginis* male quality, I quantified the amount of emission produced as well as other physical characteristics of individual male flies, such as mass, gall-size, and life span. I found that emission production is not correlated with gall size, male mass, or life span. However, I did observe that the ratio of compounds in the male emission changes with fly age.

Together, these results suggest that *S. altissima* plants eavesdrop on the olfactory signals of their insect antagonist and exploit these signals as indicators of impending herbivory. These findings document a new class of olfactory-mediated interactions with broad significance for the evolutionary ecology of plant-insect interactions.

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PREFACE

I published two chapters of this dissertation prior to my defense and served as first author on these chapters. Chapter two is published as: Helms AM, De Moraes CM, Tooker JF, Mescher MC (2013) Exposure of *Solidago altissima* plants to volatile emissions of an insect antagonist (*Eurosta solidaginis*) deters subsequent herbivory. *Proc Natl Acad Sci U S A* 110(1):199–204. Chapter three is published as: Helms AM, Moraes CM De, Mescher MC, Tooker JF (2014) The volatile emission of *Eurosta solidaginis* primes herbivore-induced volatile production in *Solidago altissima* and does not directly deter insect feeding. *BMC Plant Biol* 14(1):1–9.

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Chapter 1

Introduction
Eavesdropping on chemical signals: Cheaters and spies in the natural world

Abstract

Chemical signals and cues mediate a diverse array of ecological interactions among many different organisms. Some of the best-studied examples of chemical communication include pheromonal signaling among conspecific insects and floral volatiles attracting pollinators. Similar to other modes of communication such as visual or auditory, chemical communication is susceptible to eavesdropping by illegitimate receivers. Eavesdropping occurs when unintended receivers parasitize a mode of communication by perceiving and responding to signals not intended for their consumption, often negatively influencing the signaler and/or the intended receiver. Chemical eavesdropping represents an interesting evolutionary challenge as organisms are faced with the conflicting selection pressures of making sure their signals attract attention from the intended recipients as well as keeping their messages private from illegitimate receivers. This review highlights examples of eavesdropping on chemical signals from a wide variety of organisms and ecological contexts. I also discuss hypotheses for how chemical eavesdropping might have evolved and its potential evolutionary consequences.

Introduction

Communication among organisms is an important aspect of many species interactions and can be essential for mediating symbiotic and social relationships in nature (1–7). Communication occurs when an organism releases a physical signal conveying information into the environment and an intended recipient perceives that signal and responds to it, such that it benefits the signaler and generally also the receiver (8, 9). A mutual benefit, on average, from this transfer of information is thought to be important for maintaining evolutionary stability in communication (10).

A wide variety of signaling modalities for communication have evolved including acoustic, visual, electric, tactile, and even chemical signals (11–16). Such signals may be used to communicate in a variety of contexts, ranging from mate attraction to warning conspecifics and are typically under selection to improve their reliability and transmission efficiency (8–10, 17). However, signalers and their intended recipients are not the only organisms in an environment and other organisms can often exploit intraspecific signals (18). Eavesdropping occurs when unintended recipients parasitize a mode of communication by perceiving and responding to signals not intended for them (Fig. 1). This typically, although not necessarily, negatively influences the signaler and/or intended receiver (8, 9, 14, 18, 19).

Many organisms also perceive physical cues from other organisms and benefit from these cues at the cost of the emitter. One example is prey avoidance of predator odors (20, 21). Cues can also transmit information, however they are typically unintentional by-products of an organism's behavior or physiology, whereas signals are

shaped by natural selection with the purpose of conveying information to benefit an emitter and intended recipient (10, 16). Although some cues are superficially similar to signals, for this review, I will limit the focus to parasitism of ecological signals and will not include examples of cue-perception.

Eavesdropping is expected to occur if it increases the amount of information an organism can glean from its surroundings, such that it increases an individual's survival or fitness (22). To date, many examples of eavesdropping in nature have been documented, including a variety of signaling modalities (14, 18). Some of the best-studied examples include predators eavesdropping on visual and auditory signals and exploiting these to locate prey (14, 18, 23–25). A well-known example of eavesdropping on visual signals occurs in male fireflies. Male fireflies seek mates by emitting bioluminescent flashes while in flight. Several predatory species eavesdrop on signaling males, including bats, nocturnal birds, and female fireflies of other species (12, 26, 27). Calling male crickets represent an example of eavesdropping on auditory communication. Most male crickets emit a chirping call to attract females and repel other males. Predators and parasitoids eavesdrop on the calls of male crickets to locate both male and female prey (14, 28–30).

Chemical signals also represent an important signaling modality in nature, mediating interactions among many taxa including animals, plants, bacteria, and fungi (2, 3, 5, 31–34). Despite their importance, however, the study of chemical signals is relatively young, with the first examples of pheromones and "Schrekstoffe," or alarm signals identified within the past one hundred years (35–37). As with other modes of communication, chemical signals are also susceptible to eavesdropping. In this review, I

discuss examples of eavesdropping on chemical signals from a variety of organisms and ecological contexts. I also examine potential hypotheses for how eavesdropping on chemical signals may have evolved and what the evolutionary consequences of such eavesdropping might be.

Chemical signals

Chemical communication in nature is widespread in both terrestrial and aquatic ecosystems and to date numerous chemical signals have been identified among a diverse array of organisms (38–44). The chemical properties of these signals are also diverse ranging from volatile compounds to water-soluble peptides or solid cuticular hydrocarbons (45–50). An important point to consider is that chemical signals are typically classified according to their function or effect in specific interactions, because a single compound might be produced by several species and can perform different functions depending on the ecological context (51).

Information-conveying chemicals or "infochemicals" are typically grouped into two categories based on whether the information exchange is inter-species or intra-species specific. An infochemical that mediates an interaction between two individuals of different species is called an allelochemical, whereas an infochemical mediating interactions between two individuals of the same species is called a pheromone (35, 51, 52). Many different functions have been identified for allelochemicals and pheromones, such as attraction, repulsion, warning, and defense (42, 46, 52–54).

Particularly well-known examples of allelochemical-mediated communication are pollinator attraction to floral volatiles (55) and various types of pheromonal communication among conspecific insects. Sex pheromones are chemical signals emitted by one sex of a species to attract potential mates (38, 42, 45, 52, 56–58). Alarm pheromones are infochemicals released in response to danger or predation to warn conspecifics or reduce predator success (36, 46, 59). Aggregation pheromones are emitted to recruit conspecifics of both sexes and may be involved in mate selection, resource exploitation, or defense against predators (60, 61). Pheromones for marking territories are another common chemical signal (45, 53, 54). And trail pheromones are commonly associated with social-insect foraging behavior and serve as both a recruitment signal and navigational guide to a resource (62, 63).

Another important group of chemical signals that does not fit neatly into one of these categories is plant volatile signals. Plants emit volatile compounds in response to herbivore feeding, pathogen infection, and mechanical damage (34, 64–67). The prevailing theories are that damage-induced plant volatiles evolved as both an anti-microbial defense to prevent infection of wounded tissue, and an intra-plant signaling mechanism to overcome vascular constraints (64, 67). In addition to their role in intra-plant signaling, herbivore-induced plant volatiles are also allelochemicals that attract predators and parasitoids (48, 55, 68–70). It is likely that this relationship evolved secondarily and is now under positive selection because of the mutual benefit for plants receiving assistance with defense against herbivores and predators receiving information to help them locate prey. Recent evidence suggests that not only terrestrial but also

aquatic plants use allelochemicals to recruit predatory species to aid in their defense (38, 71).

Eavesdropping on chemical signals

Chemical signals contain information that is valuable not only for the intended receiver, but also for other organisms in the environment. Eavesdropping occurs when unintended recipients perceive and benefit from signals meant for others, often negatively affecting the emitter and/or intended recipient (8, 9, 14, 18, 19). Eavesdropping can be interceptive, where an individual intercepts a signal intended for another individual, or social, where individuals gather information on other individuals by observing their signaling interactions with conspecifics (19). Information obtained through eavesdropping can reveal details about conspecifics or the presence of potential prey, predators, and competitors. Here I will discuss examples of eavesdropping on some types of chemical signals described in the previous section, with the majority of examples presented here representing interceptive eavesdropping.

Sex pheromones

Sex pheromones tend to be highly specific and comprise finely tuned ratios of chemical compounds that attract conspecifics but not members of closely related species (72, 73). Despite or perhaps because of this specificity, many organisms have evolved the ability to eavesdrop on sex pheromones, likely because these signals contain fairly

reliable information about the emitter (14, 52, 74). Sex pheromones are commonly exploited by eavesdropping predators and parasitoids searching for suitable prey or hosts. Several studies have documented attraction of wasps in the family Trichogrammatidae to the sex pheromones of their host insects. These egg parasitoids locate signaling adult insects, travel phoretically to oviposition sites, and then parasitize freshly laid eggs (14, 52, 74–77). This is likely an adaptive strategy for the parasitoids to efficiently locate otherwise cryptic eggs in the correct developmental state for successful parasitism (78). Another parasitoid wasp, *Aphidius colemani* is attracted to the sex pheromone of its aphid hosts, which is a unique strategy given the often parthenogenic reproduction of aphids (79, 80). Dipteran parasitoids also eavesdrop on sex pheromones to locate their hosts, including the tachinid parasitoid (*Trichopoda pennipes*), which is attracted to the sex pheromone of male stinkbugs (*Nezara viridula*) (14, 74, 81). Spiders represent another unique example of eavesdropping on sex pheromones because of the cannibalistic behavior of female spiders toward potential mates. Male wolf spiders (*Rabidosa punctulata*) perceive the pheromone coated dragline silk from female spiders of other species and alter their courtship tactics to avoid predation (82).

Adding to these examples, a recent discovery revealed that plants can also exploit insect sex pheromones as warning cues. Tall goldenrod plants (*Solidago altissima*) perceive the putative sex pheromone of the specialist gall inducing fly (*Eurosta solidaginis*) and respond by enhancing their anti-herbivore defenses (83, 84). This finding represents the first documented example of plants perceiving insect-derived odors and is one of only a handful of examples of cross-kingdom chemical eavesdropping.

Alarm pheromones

Many predators and parasitoids eavesdrop on the alarm pheromones of their prey. Aphid predators and parasitoids, spanning several orders, exploit aphid alarm pheromones to locate prey (46, 85, 86). Ants also emit alarm pheromones when threatened and these pheromones are also subject to eavesdropping by and predators and parasitoids, including phorid flies and a zodariid spider (46, 87–89). While these signals provide conspicuous, reliable information about the location of prey, the release of alarm pheromones might also indicate potential competition with other predators and parasitoids. In some cases, however, it can also indicate reduced pressure from enemies. Gravid lady beetles are attracted to alarm pheromones of the scale-tending ant (*Azteca instabilis*). When ants are attacked by phorid flies, they emit an alarm pheromone and enter a temporary motionless state, thus giving female lady beetles an opportunity to oviposit on the undefended scale insects (90).

Aggregation and recruitment pheromones

Aggregation and recruitment pheromones are also susceptible to eavesdropping by predators, parasitoids, and competitors. Because these pheromones recruit groups of conspecifics, they represent a potentially important source of information about the presence of prey or competitors. Bark beetles are a well-studied example of insect aggregation pheromones. After locating a suitable host tree, bark beetles emit aggregation pheromones to recruit conspecific beetles to aid in the attack and overcome the tree's defenses (61). Predatory histerid and clerid beetles are attracted to bark beetle

aggregation pheromones, exploiting these signals to locate their prey (91, 92). More recent studies have found that some parasitoids also eavesdrop on the aggregation pheromones of their dipteran and lepidopteran hosts. The parasitoid wasp *Leptopilina heterotoma* is attracted to the aggregation pheromone of its host *Drosophila melanogaster*, resulting in increased parasitism risk when maggots aggregate to better exploit a food resource (60). Parasitoids of the codling moth (*Cydia pomonella*) also eavesdrop on their host's aggregation pheromone. The wasp *Mastrus ridibundus* is attracted to the pheromone emitted by cocoon-spinning codling moth larvae that is intended to attract conspecifics to pupation sites (93).

Stingless bees eavesdrop on recruitment pheromones of other stingless bee species to gain information about their competitors. One aggressive species, *Trigonia spinipes* detects and orients toward the recruitment pheromone of its competitor *Melipona rufiventris*, killing or driving away *M. rufiventris* and taking over the food source (94). However, another less aggressive species *Trigonia hyalinata*, exhibits avoidance behavior when exposed to the recruitment pheromone of its aggressive competitor *T. spinipes* (95). This finding is noteworthy because it documents an example of eavesdropping with a potential benefit for the emitter and suggests selection for more conspicuous signaling (96).

Trail pheromones

The trail pheromones of some social insect species are subject to exploitation by competitors and parasitic species. Similar to the previous example of stingless bees, giant

Asian honey bees (*Apis dorsata*) detect and avoid the trail pheromones of foraging weaver ants (*Oecophylla smaragdina*) to prevent aggressive encounters. These sympatric species utilize the same host plants and weaver ants often kill honey bees in competitive interactions (97). The parasitic ant species *Cephalotes specularis* visually mimics its hyperaggressive host species *Crematogaster ampla* and actively avoids contact with foragers by detecting and avoiding their trail pheromone (98). Competing ant species also detect heterospecific trail pheromones to share trails while competing for food resources (99).

Plant volatiles

Plant volatiles are another group of chemical signals frequently parasitized by eavesdroppers. Plants emit characteristic blends of volatile compounds following insect damage. As previously discussed, these herbivore-induced plant volatiles (HIPVs) benefit emitting plants by recruiting predators and parasitoids that attack feeding herbivores (48, 68, 69, 100). Additionally, HIPVs are important intra-plant signals for plants under attack to overcome limited vascular connectivity and regulate defenses in distant tissues (64, 101–103). However, a downside to plant-volatile signaling is the highly conspicuous nature of these information-rich signals, which has led to eavesdropping by other organisms. Undamaged plants also perceive the volatiles emitted by their insect-damaged neighbors and respond by priming or enhancing their own anti-herbivore defenses. This phenomena has been documented in many plant species including agricultural plants such as maize (*Zea mays*) and long-lived woody shrubs like sagebrush (*Artemisia tridentata*)

(64, 104, 105). Some plants also eavesdrop on volatile signals emitted by other plant species (106, 107). This eavesdropping by neighboring plants can have negative consequences for emitting plants if it increases the fitness of their competitors; however, the negative effects might be ameliorated if the signals are intercepted by their kin (64, 108).

Plant volatiles are also subject to eavesdropping by insects. Some herbivores and florivores eavesdrop on floral volatiles of their host plants. For example, cucumber beetles (*Acalymma vittatum*) are attracted to the floral scents of *Cucurbita* blossoms and floral volatiles of thistles in the genus *Cirsium* attract several taxa of folivorous insects (109, 110). Herbivore-induced plant volatiles may also increase the risk of further herbivore attack for some plants. Colorado potato beetles (*Leptinotarsa decemlineata*) are attracted to the odors of insect-damaged potato plants and *Spodoptera litttoralis* larvae are attracted to herbivore-induced volatiles from maize plants (111, 112). However, some insects also avoid the odors of insect-damaged plants. Gravid female moths avoid ovipositing on damaged plants where their offspring would face greater competition as well as potentially higher levels of induced plant defenses (113).

Internal plant signals

Internal plant chemical signals can also be involved in eavesdropping. Larvae of the corn earworm (*Helicoverpa zea*) induce expression of cytochrome P450 genes associated with detoxification in response to detection of the plant defense phytohormones jasmonate and salicylate. Eavesdropping on internal plant signals allows

the caterpillars to prepare their detoxification enzymes against induced plant chemical defenses (114, 115). A recent study also found that plants eavesdrop on internal signals of their insect-damaged neighbors when connected by common underground mycelial networks of mycorrhizal fungi (116).

Additional examples of chemical eavesdropping

Numerous additional examples of eavesdropping on chemical signals have been identified that include nematodes, fungi, mammals, bacteria, and aquatic arthropods. Nematode pheromones are susceptible to eavesdropping by a fungal parasite as well as other nematodes. Nematophagous fungi perceive ascaroside dauer pheromone to locate their nematode prey and nematodes themselves perceive the ascaroside dispersal pheromones of other nematode species (117, 118). Olfactory communication is also important for mammals and there is evidence that predators eavesdrop on territorial scent markers of their mammalian prey (119). To date only a few examples of eavesdropping on chemical signals have been identified in aquatic systems. This is not due to a scarcity of chemical communication in aquatic systems, but more likely because of the difficulty of isolating and identifying waterborne chemical cues. Two examples of aquatic chemical eavesdropping include the green seaweed *Ulva* perceiving bacterial signaling molecules used in quorum sensing to select suitable attachment sites (120) and female crayfish (*Procambarus clarkia*) that eavesdrop on visual and chemical signals of fighting male crayfish to select the odor of the dominant male (121, 122).

Evolution of eavesdropping on chemical signals

When we consider the evolution of eavesdropping in general, two major questions come to mind. First, "How does eavesdropping evolve?" and second, "Which signals are most susceptible to eavesdropping?". In this section I will address these two questions and make predictions about the evolution of eavesdropping on chemical signals.

In the few examples presented in the previous section where eavesdropping appears to benefit the emitter as well as the unintended receiver, we can easily conceptualize how natural selection might favor (or at least not select against) this transfer of information, and thus allow the eavesdropping to persist. One might even argue that this information transmission is a form of communication because of the net benefit for both parties. In fact, by some narrower definitions, such a situation is not considered true eavesdropping, which must have a negative effect on the signaler (8). For situations that follow this narrower definition of eavesdropping, where an illegitimate receiver intercepts a signal at a cost to the signaler, we can develop criteria necessary for eavesdropping to evolve and persist. For eavesdropping to be evolve, I predict:

1. The signal must contain meaningful, reliable information that benefits the unintended receiver.
2. The emitted signal must be detectable by the unintended receiver.
3. Despite eavesdropping, communication using this signal must confer an advantage on the emitter.

To meet the first condition, perceiving a signal should transmit valuable and reliable information to the eavesdropper. To be evolutionarily stable, the unintended

recipient should not respond to the signal unless it carries information of value (10). In communication, signals are typically under selection to improve their reliability and transmission efficiency for intended recipients, also making them more reliable and detectable for unintended recipients (8–10, 17).

The second important condition for eavesdropping to evolve is that a potential eavesdropper must be able to perceive the signal. There are two aspects necessary to meet this condition. First, a signal must be available for perception and, second, an organism must possess the physiological ability to perceive the signal. It has been suggested that signals transmitted over long distances, or those that leave extended spatial traces, are most susceptible to eavesdropping (94). As discussed previously, selection acts on signals to increase their conspicuousness to intended receivers for a higher probability of successful transmission, ensuring the signal is available for the eavesdropper (8–10).

Evolution of the physiological ability to perceive and process a signal presents another challenge. All organisms possess some sensory abilities with which they process information about their environments. In some cases, a potential eavesdropper likely already possesses the necessary machinery to perceive a particular kind of signal (e.g. auditory, visual, chemical) and only slight modification is necessary to coopt that machinery for eavesdropping on a new signal. An example of this is the evolved sensitivity of predatory clerid beetles to the pheromone of their bark beetle hosts (123). In other cases, however, organisms have evolved entirely novel perception abilities for eavesdropping. One such example is the evolution of tympanal hearing organs in tachinid parasitoids. Flies in the tribe Ormiini have evolved an organ that allows them to eavesdrop on the songs of their calling orthopteran hosts (14, 124). We know natural

selection is capable of producing complex sensory organs such as eyes by working through slow, incremental changes over time (125); thus it is conceivable that natural selection can also produce novel sensory organs for eavesdropping.

The third condition refers to the persistence or stability of eavesdropping. Communication by definition is a transfer of information that is, on average, beneficial to the emitter of the signal. If the transmission of information does not benefit the signaler, natural selection would favor those individuals who do not signal. However, if transmitting information is generally beneficial for an organism (i.e. the risk of eavesdropping is not too high) or is essential to an organism's survival or reproduction, we would expect positive selection for signaling (8). This benefit for the signaler ensures the availability of signals for potential eavesdroppers.

When we consider if a signal might be susceptible to exploitation by eavesdroppers, there are four aspects to consider: 1) value of information to the eavesdropper, 2) reliability of the information, 3) conspicuousness of the signal, and 4) value of successful signal transmission for the emitter. In the case of chemical signals, we can use these criteria to consider which signals are most likely to experience eavesdropping. Although evolution may favor individuals whose signals are as inconspicuous as possible, it is impossible for a species that relies on chemical communication to completely avoid emitting chemical signals. Thus, infochemicals such as pheromones that are good indicators of the presence of an organism, are available for predators and parasitoids to exploit (74). Sex pheromones represent a chemical signal that appears especially likely to experience eavesdropping and indeed numerous examples have been documented (14, 74, etc.). In most cases, production of sex pheromones is

intrinsically linked to reproductive success and is under selection to provide honest, reliable signals (42, 126). Additional work is needed to decipher the fitness benefits for communication using particular types of chemical signals and whether any are more or less susceptible to eavesdropping.

Evolutionary consequences of chemical eavesdropping

One evolutionary consequence of eavesdropping, according to the definition that eavesdropping negatively affects the signaler, is conflicting selection pressures for inconspicuous and yet successfully transmitted signals. This has led, in several cases, to unique adaptations for escaping eavesdropping. We can think of the relationship between signalers and eavesdroppers as a co-evolutionary arms race where signalers attempt to escape eavesdropping, while eavesdroppers counter with new adaptations to overcome these escape attempts. If there is a sufficient benefit for a potential eavesdropper to perceive a signal, that signal will only remain private if sufficient evolutionary barriers exist to make it unlikely for a potential eavesdropper to evolve an ability to detect it (18).

A few different strategies for preventing eavesdropping on chemical signals have been identified. The first is selection for less conspicuous signals. In many insect mating systems, especially among Lepidoptera, females release tiny amounts of pheromones and highly sensitive males follow the pheromone plume to find mates (127). This is thought to reduce the conspicuousness of the chemical signal and reduce predation risk for female signalers. Another strategy is to change the chemical signal itself. Changes in pheromone chemistry in response to predator and parasitoid pressure have been observed for both

bark beetles and stinkbugs (128, 129). The pine engraver (*Ips pini*) has evolved a preference for a different chiral blend of its pheromone ipsdienol compared to its predator, *Thanasimus dubius*, allowing the bark beetles to escape eavesdropping and subsequent predation (128). Differences in pheromone blends of the southern green stink bug (*Nezara viridula*) have been observed among populations across several geographic regions with varying parasitism rates, suggesting that pheromone blends have shifted in response to parasitism by tachinid flies (129). Finally, in some cases, organisms have evolved adaptations to avoid emitting a signal all together. Some insects feed on plant fruits instead of their vegetative tissue to prevent the plants from releasing herbivore-induced volatiles and thus signaling to natural enemies (130).

Of note, however, is the example of eavesdropping by stingless bees on the recruitment pheromone of another species that resulted in a net benefit for both parties (95, 96). Here the authors argue that this case of eavesdropping selects for more conspicuous signals because it allows the bees to avoid aggressive interactions and forage more efficiently. As discussed above, however, this example might be more accurately described as communication, where the recruitment pheromone of one bee species has been coopted as an allelochemical for communicating with the other species.

Conclusions

Chemical communication is widespread in nature and chemical signals are susceptible to eavesdropping by unintended receivers. Eavesdropping has been documented for a variety of different types of chemical signals, including both

pheromones and allelochemicals. Many different taxa including plants, animals, bacteria, and fungi all participate in chemical eavesdropping. For eavesdropping on chemical signals to evolve and persist, a signal must contain valuable information, be perceivable, and communication with that signal must remain beneficial for the signaler. We would expect eavesdropping to occur most frequently on signals that are informative, conspicuous, reliable, and unavoidable. Because of the cost to signalers, strategies have evolved to avoid perception of signals by unintended receivers.

Figures

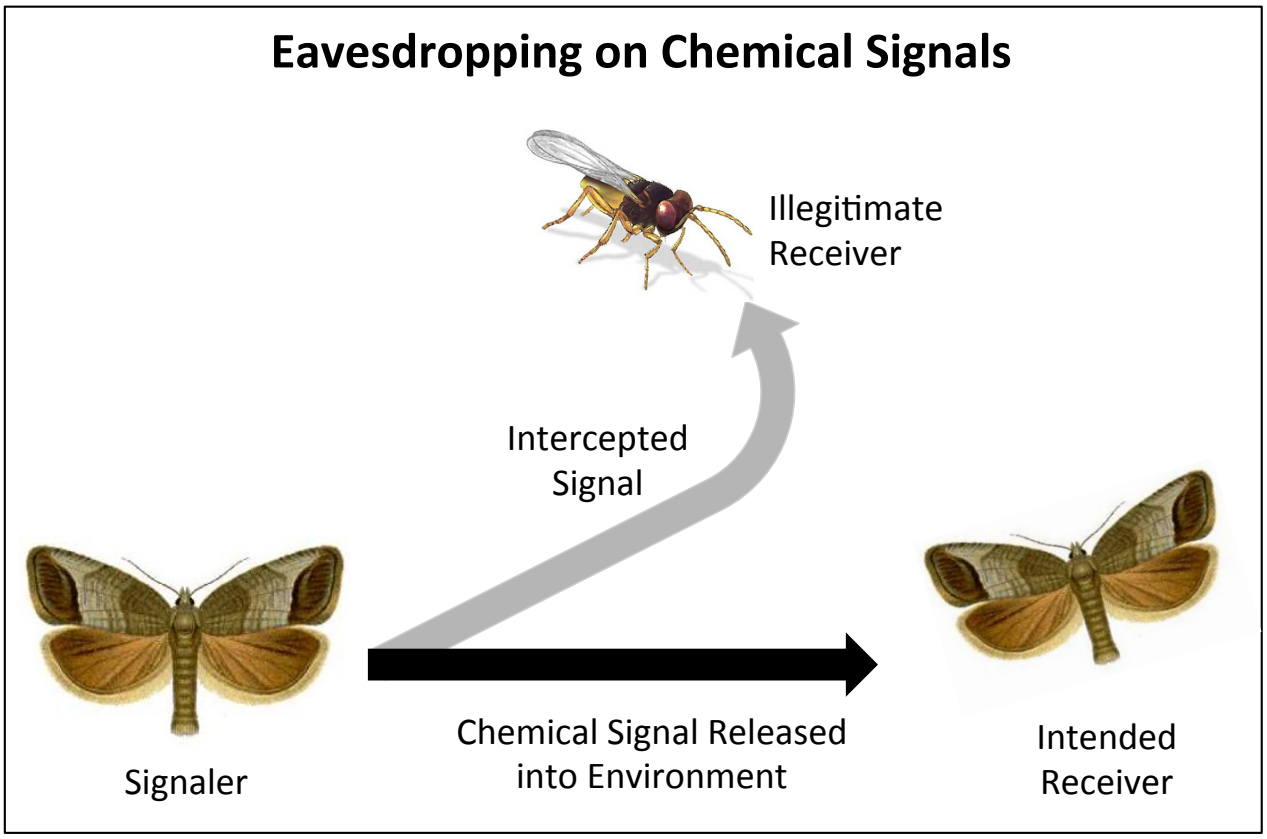

Figure 1-1
A signaling moth releases a pheromone into the environment to attract a potential mate. An eavesdropping parasitoid perceives and exploits the signal to locate its moth host.

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Chapter 2

Exposure of *Solidago altissima* plants to volatile emissions of an insect antagonist (*Eurosta solidaginis*) deters subsequent herbivory

Abstract

Recent work indicates that plants respond to environmental odors. For example, some parasitic plants grow toward volatile cues from their host-plants, and other plants have been shown to exhibit enhanced defense capability following exposure to volatile emissions from herbivore-damaged neighbors. Despite such intriguing discoveries, we currently know relatively little about the occurrence and significance of plant responses to olfactory cues in natural systems. Here I explore the possibility that some plants may respond to the odors of insect antagonists. I report that tall goldenrod (*Solidago altissima)* plants exposed to the putative sex attractant of a closely associated herbivore, the gall-inducing fly *Eurosta solidaginis*, exhibit enhanced defense responses and reduced susceptibility to insect feeding damage. In a field study, egg-laying *E. solidaginis* females discriminated against plants previously exposed to the sex-specific volatile emissions of males; furthermore, overall rates of herbivory were reduced on exposed plants. Consistent with these findings, laboratory assays documented reduced performance of the specialist herbivore *Trirhabda virgata* on plants exposed to male fly emissions (or crude extracts), as well as enhanced induction of the key defense hormone jasmonic acid in exposed plants following herbivory. These novel findings from a classic ecological study system document a previously unexplored class of plant-insect interactions involving plant responses to insect-derived olfactory cues.

Introduction

Olfactory cues and signals play important roles in a diverse array of ecological interactions among plants and insects. The best documented of these interactions include pheromonal signaling between conspecific insects and the utilization of plant-derived odors as foraging cues by insect pollinators, herbivores, and predators (1-7). Recent findings demonstrate that plants themselves can also perceive and respond to environmental odors. For example, parasitic plants in the genus Cuscuta utilize host-derived volatiles to direct their growth towards preferred host plants (8)—apparently utilizing host-plant odors as foraging cues in much the same way that insect herbivores do (5, 6). In other systems, plants appear to perceive the characteristic odors emitted from herbivore-damaged plant tissues as warning cues indicating the presence of potential attackers (9-11). Thus, the perception of volatile chemical cues appears to play important roles in plant ecology, though our understanding of the prevalence and significance of plant olfaction in natural systems remains quite limited.

Most previous work on plant responses to odor cues has addressed "priming" of induced defense responses. Plants frequently employ defenses that are induced by environmental stimuli, rather than being expressed constitutively, in environments where the occurrence of particular antagonists (e.g. herbivores and pathogens) is not entirely predictable—presumably to conserve resources and maintain the flexibility to precisely target defense responses against specific attackers (12). Still further economy may be achieved through priming responses, in which induced defenses are made ready for deployment in response to cues reliably associated with impending attack (10, 11, 13, 14).

Priming of plant defenses by olfactory cues has previously been documented following exposure to herbivore-induced volatiles emitted either by neighboring plants (9, 10) or by other parts of the same plant (11, 14) (the latter finding giving rise to speculation that such mechanisms may have initially evolved to overcome constraints on the within-plant transmission of wound signals imposed by the discontinuous architecture of plant vascular systems, with eavesdropping by neighboring plants arising secondarily [11]).

Defense priming has also been reported in response to (non-olfactory) cues directly associated with the presence of herbivores, including insect footsteps on leaves and broken trichomes (15, 16). However, direct plant perception of insect-derived olfactory cues has not previously been reported, despite many herbivores emitting volatile chemicals that function in intraspecific communication (e.g., sex, aggregation, and alarm pheromones [1-3]) or defense (e.g., predator repellents [17]). Furthermore, these compounds are frequently released in substantial quantities and in proximity to plants on which feeding will subsequently occur (18, 19); thus, they would appear to provide a class of potentially reliable olfactory cues that plants might profitably utilize for defense priming or induction.

In light of these observations, the current study explored whether and how the anti-herbivore defenses of tall goldenrod, Solidago altissima L., are influenced by exposure to chemical emissions of its specialist herbivore Eurosta solidaginis (Fitch), a tephritid fruit fly (Fig. 1A) whose larvae induce ball-shaped galls in the stems of this plant species (Fig. 1B). The interactions of these two species have been studied for decades, and suggest a tightly co-evolved relationship (20, 21). Moreover, gall induction and feeding by E. solidaginis greatly reduce S. altissima growth and fitness (21),

implying that individual plants may benefit from efficient deployment of effective defenses.

I explored whether the chemical emissions of male E. solidaginis might induce or prime anti-herbivore defenses in S. altissima, as the ecology of this system suggests that these emissions may provide a salient cue reliably associated with impending attack. Male E. solidaginis begin to emerge prior to females (during mid-May in the Northeastern US), and following emergence typically perch on the upper leaves of S. altissima ramets, often for hours at a time (20, 21), and emit copious amounts (~70 ± 20 [SD] μg over 24 h) of a volatile blend that I hypothesize to function as a sex attractant. (Through GC-MS analysis, the blend was found to be dominated by spiroacetals, whose biological significance as insect-derived volatiles remains largely undocumented, but which are known in some systems to function as pheromones, kairomones, or allomones [22].) Females begin searching for oviposition sites immediately after mating and have been observed to oviposit into stems of the same goldenrod genet on which mating occurs (frequently within 30 minutes [20]). Eggs typically hatch 5-8 days later (20, 21), and galls become visually apparent within three weeks. It thus seems plausible that exposure to the volatile emissions of male flies might reliably predict subsequent oviposition and larval herbivory. I therefore undertook field and laboratory experiments designed to explore the ecological role of the E. solidaginis emission and, in particular, its potential influence on the defense responses of S. altissima.

Results

Volatile emissions from male *E. solidaginis* flies are attractive to female flies.

In initial olfactometry assays testing the attractiveness of male and female flies to the odors of the opposite sex, I found male flies (which, as described above, more or less continuously release copious amounts of a volatile blend dominated by spiroacetals) to be significantly attractive to females; in contrast, female flies were not significantly more attractive to males than clean air (Table 1). While not sufficient to formally characterize the male emission as a sex pheromone, these findings are consistent with my hypothesis that the emission plays a role in mate attraction.

Plant exposure to volatile-emitting *E. solidaginis* males reduces subsequent rates of ovipuncture by *E. solidaginis* females and overall levels of herbivory in the field.

To test the hypothesis that exposure to the male E. solidaginis emission enhances S. altissima defense responses, I conducted a large-scale field study comparing the oviposition preferences of E. solidaginis females for plants given prior exposure to male E. solidaginis or various controls; I also assessed overall rates of herbivory on these plants. I found that randomly selected S. altissima plants on which male E. solidaginis were caged for three days subsequently experienced significantly reduced rates of ovipuncture by E. solidaginis females (i.e., insertion of the ovipositor into plant tissues as assessed by characteristic patterns of tissue damage [20, 21, 23, 24]) compared to control plants similarly exposed to either (i) female E. solidaginis, (ii) common house flies, or (iii) empty cages (Fig. 2A; Chi-squared test of independence, $X^2_3 = 12.5$, $P = 0.006$). (The

female E. solidaginis and housefly controls were designed to account for the effects of fly-associated cues other than the male volatile emission). The proportion of ovipunctured plants observed in the control treatments was 3.7 - 4.8 times higher than that observed for plants exposed to E. solidaginis males, whereas no significant differences were observed among the three control treatments (post-hoc relative risk analysis assessing risk of each control group receiving an ovipuncture relative to the male E. solidaginis treatment [with associated 95% confidence intervals]: male vs. female: 3.80 [1.35-10.65]; male vs. house fly: 4.75 [1.73-13.03]; male vs. empty net: 3.68 [1.31-10.39]). Thus, egg-laying E. solidaginis females appear to actively discriminate against plants previously exposed to male flies—female E. solidaginis are known to assess host-plant quality prior to oviposition by tasting bud tissue with chemoreceptors located on their feet and mouthparts and often reject more resistant plant genotypes (21, 24, 25). Because E. solidaginis females so rarely oviposited on exposed plants in my field study the data obtained do not allow us to assess the effects of exposure on gall development following oviposition. However, levels of foliar damage by chewing and leaf-mining insects were dramatically reduced on plants previously exposed to E. solidaginis males compared to each of the control groups (Fig. 2B; Negative binomial regression: male vs. female: $t_{1,109} = 3.794$, $P = 0.0002$; male vs. house fly: $t_{1,106} = 2.837$, $P = 0.005$; male vs. empty cages: $t_{1,107} = 3.236$, $P = 0.002$), suggesting that the reduced preferences of E. solidaginis females for exposed plants may reflect changes in plant quality that also deter other insects.

S. altissima plants exposed to male _E. solidaginis_ volatile emissions are less palatable to specialist herbivores.

I further explored the effects of E. solidaginis emissions on S. altissima defenses through controlled insect feeding assays conducted in the laboratory. Because the galling habit of E. solidaginis larvae limits their usefulness in such assays, I assessed effects of volatile exposure on the performance of adults and larvae of another S. altissima specialist, the goldenrod leaf beetle (Trirhabda virgata; Fig. 3A), a member of the herbivore community present at my field sites. Previous studies have documented a negative association between E. solidaginis and T. virgata in the field (26) and shown that T. virgata feeding deters subsequent oviposition by E. solidaginis (27), suggesting potential overlap in the defenses deployed against these two herbivores.

Consistent with my observation of reduced herbivory on emission-exposed plants in the field, adult T. virgata consumed significantly less leaf tissue during 24 h of feeding on plants exposed to live male flies (over the preceding 24 h) compared to control plants (Fig. 3B; ANOVA $F_{1,11} = 9.32$, $P = 0.012$), indicating a reduction in palatability following exposure. To confirm that the reduced herbivory observed in my emission treatment was not explained by some other cue associated with presence of the flies, I repeated this experiment using biologically realistic doses of crude extracts of the E. solidaginis volatile emission. This design yielded results similar to those of the previous experiment: beetles feeding upon emission-exposed plants ate significantly less plant tissue than those feeding upon control plants (Fig. 3C; ANOVA $F_{1,9} = 5.60$, $P = 0.046$), confirming that exposure to the volatile emission itself, in the absence of any other fly-derived cues, induced changes in the plants that deterred feeding. (I therefore used live

flies in subsequent experiments to mimic as closely as possible the exposure occurring in nature.)

Next I conducted a similar performance assay with T. virgata larvae (Fig. 3D) rather than adults—the larvae are active and feeding during the period when E. solidaginis females mate and oviposit, and both insect species appear to prefer the most vigorous-growing S. altissima ramets (24). As with adult beetles, larvae consumed far less leaf tissue on plants with prior exposure to fly volatiles than on unexposed controls (Fig. 3E).

S. altissima plants exposed to male _E. solidaginis_ volatile emissions exhibit more vigorous defense responses.

To determine whether the lower levels of herbivory observed on volatile-exposed plants were mediated by enhanced plant defense responses, I assayed levels of the key defense-related phytohormone jasmonic acid in exposed and unexposed plants before and after (similar amounts of) feeding by adult T. virgata. Jasmonic acid is a key defense phytohormone that regulates the expression of genes involved in defenses against herbivores, and up-regulation of jasmonic acid is frequently assayed as in indicator of defense induction (10, 28). Prior to herbivory, volatile-exposed and control plants had similar jasmonic acid levels, but six hours after the initiation of feeding, exposed plants exhibited significantly higher concentrations of jasmonic acid than controls (Fig. 4; repeated measures ANOVA: time factor: $F_{1,23} = 80.0$, $P < 0.00001$; treatment*time interaction: $F_{1,23} = 7.4$, $P = 0.021$), indicating that exposure to the male E. solidaginis emission enhanced jasmonic acid-mediated defense responses to subsequent herbivory.

Volatile emissions from non-coevolved insect species do not similarly influence plant defenses.

As a further test of the hypothesis that the enhanced defense responses of S. altissima represent a specific reaction to the emission of its coevolved herbivore, I assessed (as a separate treatment in the larval feeding experiment) the effect of exposure to the sex pheromone of an unassociated herbivore, the western bean cutworm (Striacosta albicosta [Smith]), an agricultural pest of maize (Zea mays L.) and beans (Phaseolus vulgaris L.; (29, 30). Here I found no differences in the damage inflicted by T. virgata larvae on plants exposed to western bean cutworm pheromone compared to unexposed control plants (Fig. 3E). Moreover, higher damage levels were observed both for plants treated with the western bean cutworm pheromone and for control plants compared to plants exposed to E. solidaginis emission. This result suggests that the response of S. altissima to insect-derived volatiles is not broadly tuned, but more likely represents a specific response to a co-evolved antagonist.

Finally, to account for the possibility that my results might be explained by non-adaptive "by-product" effects of plant exposure to the E. solidaginis emission, I conducted a parallel set of experiments in a different plant species, maize (Zea mays L.), that does not have an association with this fly. Here I found that the generalist caterpillar Heliothis virescens fed similarly on unexposed (control) plants and plants exposed to volatile emissions from either E. solidaginis or western bean cutworm (unexposed: 165.01 ± 37.9 cm^2 leaf tissue removed; E. solidaginis emission exposed: 195 ± 59 cm^2; cutworm pheromone exposed: 170 ± 27 cm^2; ANOVA $F_{2,23} = 0.14$, $P = 0.87$). These

results indicate that volatile cues from neither insect species induced a defensive response effective against this generalist herbivore species.

Discussion

Taken together, my results demonstrate that exposure to the volatile emissions of male E. solidaginis flies enhanced the defense responses of S. altissima plants to subsequent herbivory and reduced their attractiveness to ovipositing female E. solidaginis (Figs. 2, 3, 4). My field study revealed a 73-79% reduction in the frequency of ovipuncture by E. solidaginis females (Fig. 2A)—indicating a reduced risk of feeding damage by E. solidaginis larvae, which can greatly reduce plant fitness (21). (Following oviposition, E. solidaginis eggs typically hatch within 5-8 days (20, 21), and previous work has documented significant mortality of early stage E. solidaginis larvae, due at least in part to plant defenses [24].) I furthermore observed a significant reduction in overall rates of foliar herbivory on emission-exposed plants in the field (Fig. 2B). Consistent with these results, herbivory by T. virgata adults and larvae was reduced by 41-62% relative to unexposed controls in laboratory assays (Fig. 3), a difference that might be expected to influence the significant ecological impacts of Trirhabda herbivory on Solidago—which include reduction in both above and belowground biomass (31). This reduction in herbivory may be explained by the priming of induced defense responses, as indicated by my observation of stronger JA induction in exposed plants (Fig. 4). Finally, the observation of no similar effects in plants exposed to the pheromones of unassociated herbivores suggests that the patterns reported here reflect

adaptive responses of S. altissima to the volatile emissions of its specialist herbivore E. solidaginis.

To my knowledge, this is the first report of plant response to an animal-derived olfactory cue, thus documenting a novel class of volatile-mediated interactions among plants and insect herbivores. As noted above, other recent work clearly demonstrates that plants can respond to environmental odors (e.g., 8-11), including by priming defense responses following exposure to herbivore-induced volatiles of their neighbors (9-11). Nevertheless, the novelty of the current findings calls for circumspection regarding the conclusions drawn. Potential alternative interpretations of my findings include (i) the possibility that the fly emission might effect a biochemical manipulation of the host plant by the fly and (ii) the possibility of insect-insect interactions mediated by retention of some components of the E. solidaginis volatile blend on plant tissues. The former hypothesis is undercut by the observation that ovipositing E. solidaginis females discriminated against emission-exposed plants in the field, strongly suggesting that the quality of these plants as hosts for the fly is compromised rather than enhanced. The latter is directly contradicted by my observation of strongly enhanced JA responses to herbivory in emission-exposed plants (Fig. 4). Furthermore, I observed no effect on feeding by a generalist insect herbivore (H. virescens) on maize plants exposed to the E. solidaginis emission, suggesting that the emission itself does not have general deterrence effects. Yet effects are observed for multiple herbivores on emission-exposed S. altissima plants, including not only female E. solidaginis and T. virgata adults and larvae, but also the other herbivore species responsible for the bulk of foliar herbivory observed in my field studies (largely lepidopteran larvae and leaf miners). Moreover, there is no obvious

adaptive rationale for these diverse insects to respond to a fly-associated cue in absence of volatile-mediated changes in host-plant quality—competing herbivores might exhibit evolved strategies for avoiding competition with E. solidaginis, but this is difficult to reconcile with the observation that oviposition by the fly itself is reduced on plants exposed to the fly emission. Thus, I think that the enhancement of plant defense responses following exposure to the emission of E. solidaginis males is by far the most parsimonious and compelling explanation for my findings.

There are, furthermore, reasons to suspect that plant detection of herbivore-derived volatile emissions may also occur in other systems. As discussed above, previous studies have described plant responses to other classes of herbivore-associated cues encountered prior to the initiation of feeding (15, 16,32). And other work demonstrates that plants can detect volatile cues from nearby damaged or undamaged plant tissues (9, 10, 11, 33). The volatile emissions of insect herbivores might likewise be expected to provide reliable information about impending herbivory, particularly if such emissions are released in proximity to potential host plants. One might furthermore expect adaptive plant responses to insect-derived olfactory cues to emerge most readily in the context of tightly co-evolved relationships, such as that between S. altissima and E. solidaginis, or in other systems where a particular herbivore accounts for a large portion of the feeding damage inflicted on a given plant species—as happens, for example, with some bark beetles and aphids (34, 35). The sex attractants of specialist herbivores would seem particularly likely candidates to serve as cues for the priming or induction of plant defense responses, as courtship and mating frequently occur on or near prospective host plants (18, 36), as would the aggregation pheromones that some specialist herbivores

utilize to recruit conspecifics (37). While plants might also prime or induce defenses in response to volatile emissions from commonly encountered generalist herbivores, I hypothesize that such responses evolve less frequently, as generalist insects tend to be more variable in host-plant selection for oviposition or feeding (18, 36). (In the current study, I found no evidence for priming of maize defenses in response to the pheromone of the generalist caterpillar Striacosta albicosta.)

In conclusion, my findings reveal a novel and apparently adaptive feature of the tightly co-evolved relationship between *S. altissima* and *E. solidaginis* that is likely to influence the outcome of interactions between them as well as broader community dynamics. If plant response to insect-derived olfactory cues is shown to be a more general phenomenon it may have widespread implications for ecology, including not only plant defense strategies but also the ecology and evolution of insect signaling systems in environments where plants potentially act as illegitimate receivers. Finally, I speculate that this new class of volatile-mediated plant-insect interactions might also have applied relevance for the management of agricultural and forest ecosystems, perhaps via general or targeted priming of plant defenses against herbivorous insect pests.

Materials and Methods

Plant and insect material

Goldenrod (Solidago altissima) plants of the same genetic background (clone 110) were grown from rhizomes in insect-free, climate-controlled growth chambers (16 h light: 8 h dark, 22°C: 21°C; 65% relative humidity (RH)). Rhizomes were collected from

an old field near State College, Pennsylvania (USA) and stored at 4°C until use. S. altissima ramets used in experiments were approximately 35-cm tall (8 weeks old). Corn (Zea mays cv. Delprim) was grown from seed in the insect-free, climate-controlled growth chambers (16 h light: 8 h dark, 25°C: 25°C; 65% relative humidity (RH)) until plants were in the three-leaf stage. Male Eurosta solidaginis were reared from overwintering galls collected from S. altissima in the vicinity of State College, Pennsylvania (USA) during winter 2010-2011. Trirhabda virgata larvae were also collected from S. altissima near State College in early May 2011 and reared at room temperature on growth-chamber-grown goldenrod until they were used in feeding trials. Adult female T. virgata were collected from the same field site from July through September 2011 and were also kept at room temperature and fed growth-chamber-grown goldenrod. Tobacco budworm (Heliothis virescens) larvae were reared from eggs in an incubator (16 h light: 8 h dark, 22°C: 20°C; 65% RH) on an artificial casein-based diet.

Olfactometry assays

Attractiveness of the odor of male and female flies was assessed using a Y-tube olfactometer with a 1.5-cm interior diameter, 18-cm long base tube and 16-cm long arms. The olfactometer was a closed system and airflow meters regulated the movement of purified air, which was pushed through Teflon tubing past twin humidifiers, then through the two glass sample chambers containing a single male or female fly and down to the arms of the Y-tube and simultaneously pulled through the base tube. Airflow through the apparatus was 0.6 L min^{-1}. Individual flies were introduced into the base of the Y-tube and responded to odors by walking upwind into one of the arms. A fly recorded a

response when it walked 6 cm up an arm, crossed a "decision line," and remained beyond that line for at least 20 sec. Flies not reaching a decision line within five minutes were recorded as "no response". Every five trials, I changed the male or female fly, rinsed the tube with acetone and hexane, and switch treatments between arms of the Y-tube.

Field ovipuncture and herbivore damage assessment

The effects of plant exposure to male E. solidaginis emissions on the subsequent oviposition preferences of E. solidaginis females (and overall rates of herbivory) were explored through a field study conducted in a naturally occurring S. altissima population near State College, Pennsylvania (USA). In early May 2012, undamaged plants of approximately equal height (31.7 $\pm$ 5.8 [SD] cm) and spaced ~4-m apart were selected. Each plant was randomly assigned to one of four groups and then, based on its group membership, was subjected either to an emission exposure treatment or one of three controls. The upper portion of each plant was contained inside a mesh net. For treatment plants, a single, newly emerged live male E. solidaginis was placed within this net and left for 72 h. For plants in the first control group one, newly emerged live female E. solidaginis was similarly confined. For plants in the second control group, I similarly confined one common house fly (Musca domestica). For the third and final control group, the mesh nets were left empty. After the 72-h exposure period, the nets were removed from all plants. Plants were then inspected weekly for four weeks for herbivore feeding damage as well as ovipuncture scars created by (naturally occurring) E. solidaginis females—insertion of the ovipositor into the terminal bud leaves characteristic and readily observable wounds (20, 21, 23, 24, 27). Herbivore leaf damage

was recorded as the number of damaged leaves per plant and included both chewing damage and leaf mines. Data presented are from the survey conducted two weeks after the exposure treatment and represent the total herbivore leaf damage accumulated during this time. These data were analyzed by fitting negative binomial regression models to compare emission-exposed plants to each of the control groups (38). (Negative binomial regression models were used in place of poisson regression models because the data did not meet the assumption of equidispersion [38].) The models were fit by regressing the number of damaged leaves per plant on treatment group. The first ovipuncture scars on S. altissima were recorded on May 17, 2012 with new scars continuing to appear until May 31, 2012. The ovipuncture data were analyzed using a chi-squared test of independence and comparing the relative risks (38).

Emission collection

The volatile emission emitted by male E. solidaginis is newly discovered. The amount of volatile compounds released by males that I report (~70 ± 20 [SD] μg) is based on 24-h headspace aerations of eight males. The male E. solidaginis volatile blend is dominated by three spiroacetals: (5*S*,7*S*)-7-methyl-1,6-dioxasopiro[4.5]decane; (*E*)-2-methyl-1,6-dioxaspiro[4.5]decane; and (*Z*)-2-methyl-1,6-dioxaspiro[4.5]decane. These compounds account for ~95% of the total emission. Identification of these compounds in the emission was achieved via coupled gas chromatography and mass spectrometry in collaboration with Hans Alborn (USDA, Gainesville, FL, US) and Wittko Francke (University of Hamburg, Germany). Further work is required to properly describe,

characterize, and verify the role of these compounds for E. solidaginis and its interaction with S. altissima.

To collect emission from male E. solidaginis flies, two male flies were aerated for 24 h inside a small ground-glass sealed chamber. Filtered air was pushed through Teflon tubing into the chamber and over the emission-producing flies at 0.6 L min^{-1}. Air was then pulled out of the chamber with a vacuum at 0.5 L min^{-1} and over a filter containing 45 mg SuperQ (Alltech Associates, Deerfield, IL, USA). The filters were eluted using 150 µl of dichloromethane and individual samples were pooled to ensure a uniform concentration of emission. The concentration of the emission extract was quantified using a gas chromatograph with a flame ionization detector.

Laboratory emission exposure treatments

Plants were exposed to the E. solidaginis emission through exposure to either live male flies or to crude extract of the emission on rubber septa. Plants exposed to live flies were enclosed inside individual glass chambers together with two recently emerged male flies for 24 h; control plants were enclosed in individual glass chambers without flies. (While the time budgets of females have been well-characterized (39), male flies have not received as much attention, perhaps because their emission has not been previously reported. Female flies spend approximately 65% of their time resting and walking on host plants (23). It seems likely that male flies spend even more of their time perching on plants while waiting for females (20, 23), thus my exposure levels are not likely to be excessively high compared to what may occur in natural settings, particularly considering that E. solidaginis can be very abundant in some fields). Each 9-L glass chamber rested

on a two-piece aluminum base, which was supported by the rim of the plant's pot. The aluminum base had a hole in the center to allow the plant stem to pass and each stem was wrapped in cotton where it passed through the hole to plug the gap between stem and base. To avoid condensation and an unrealistic concentration of emission from developing within the chambers, air was pulled through the chambers at 0.25 L min^{-1} with a vacuum attached to a manifold that split airflow equally among the chambers.

Plants exposed to crude emission extract were placed inside individual 9-L glass chambers and two rubber septa, each containing a 12-h male equivalent of E. solidaginis emission (37.5 µl crude extract) were added to the chamber. Two more rubber septa, each containing a 12-hour male equivalent of emission were added to the chamber 12 h after the first dose. The doses were split in this way to better approximate the emission exposure experienced by plants exposed to live flies and to avoid having an initial strong concentration exposure be followed by a period of weak exposure. Control plants were enclosed in individual glass chambers containing either no flies or containing rubber septa dosed with a dichloromethane solvent control.

Plants exposed to western bean cutworm pheromone (Striacosta albicosta) were placed in glass chambers each containing a synthetic pheromone lure (Suterra, Inc., Bend, OR, USA) comprised of one rubber septum containing enough pheromone for six weeks of field use (2 mg of a three-component pheromone). Given this large amount of western bean cutworm raw material, my treatment likely "overexposed" plants to this cue, likely increasing the probability that S. altissima would have responded to the pheromone if they had the capacity to do so.

Feeding assays

For feeding assays with adult beetles, female T. virgata were starved at room temperature for 24 h. Following the 24-h emission exposure period, 3 beetles were placed into each S. altissima-containing chamber and were allowed to feed on the plant for 24 h. After this 24-h period, the beetles were removed, the plants were harvested, and the insect damage on each plant was quantified. To quantify damage, leaves were scanned and the resulting images were imported into SigmaScan (Systat Software, Inc., San Jose, CA) in order to calculate the area of leaf tissue eaten and remaining. For the T. virgata larval feeding assays, 5 larvae (each less than 1 cm in length) were added to each S. altissima plant and allowed to feed for 10 h. After 10 h, the larvae were removed, and the feeding damage was quantified as described above. For the feeding assays with Zea mays, third-instar Heliothis virescens were starved for 24 h at room temperature. Two larvae were placed into each Z. mays-containing chamber and were allowed to feed for 24 h. After 24 h, the larvae were removed and the insect feeding damage was quantified using the previously described methods.

Quantification of jasmonic acid

Individual S. altissima leaves selected for jasmonic-acid analyses were similar sized and exhibited similar levels of feeding damage. A previously described protocol was employed to extract and detect jasmonic acid in Solidago altissima plants (40, 41). Briefly, carboxylic acids were derivatized to methyl esters, which were isolated using vapor-phase extraction and analyzed by coupled gas chromatography-mass spectrometry (GC-MS) with isobutane chemical ionization using selected-ion monitoring. Amounts of

JA were quantified using 100 ng of the internal standard dihydro-JA, which was derived from methyl dihydrojamonate (Bedoukian Research Inc., Danbury, CT, USA) via alkaline hydrolysis. To confirm the identity of methyl jasmonate recovered from the samples, extracts were analyzed by GCMS with electron ionization, comparing retention times and spectra with that of the pure compound. Further samples were also processed in the absence of the derivatizing agent to confirm that endogenous methyl jasmonate was minimal.

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Figures

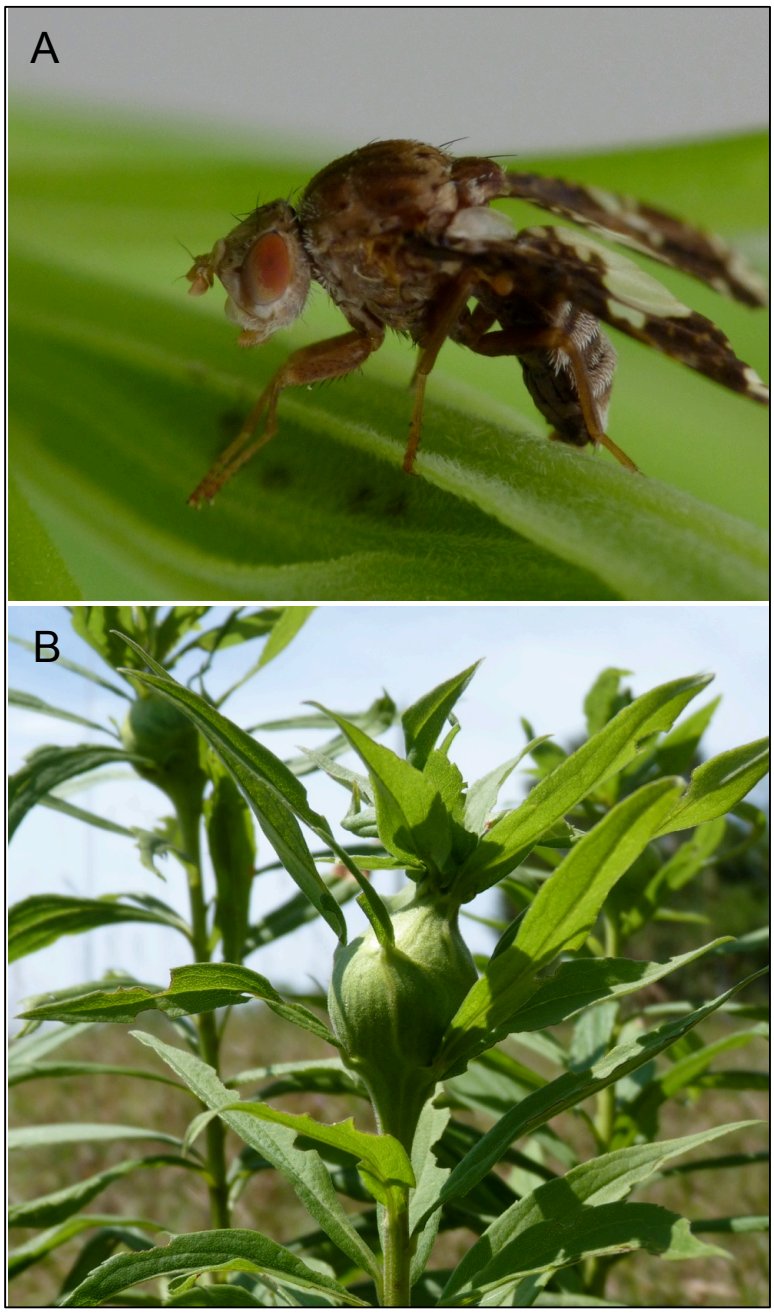

Figure 2-1 A,B
Eurosta solidaginis. (A) Male fly perching on its host plant, *Solidago altissima*, and releasing a volatile blend attractive to potential mates. (B) Developing *Eurosta solidaginis* gall on stem of *S. altissima*.

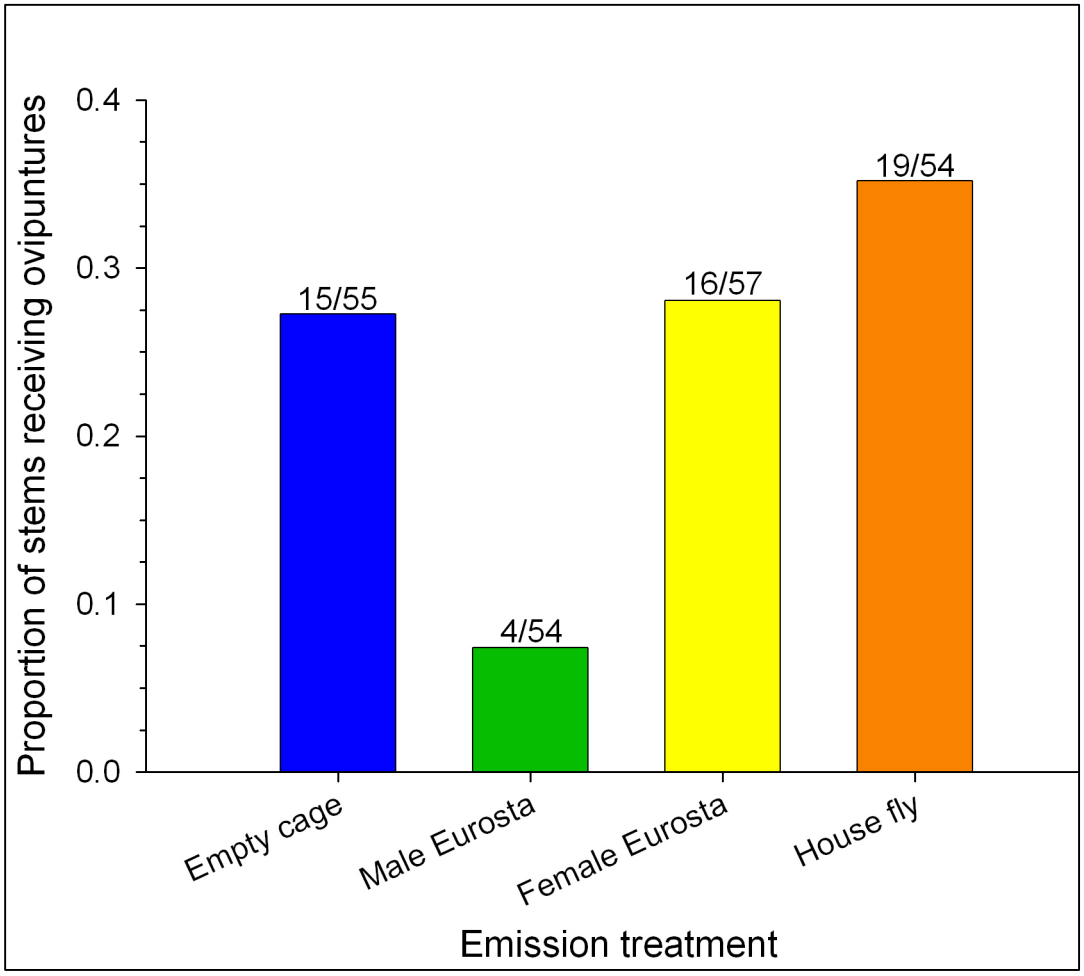

Figure 2-2
Rates of ovipuncture by *E. solidaginis* females on *S. altissima* following exposure to the male emission compared to the three control groups.

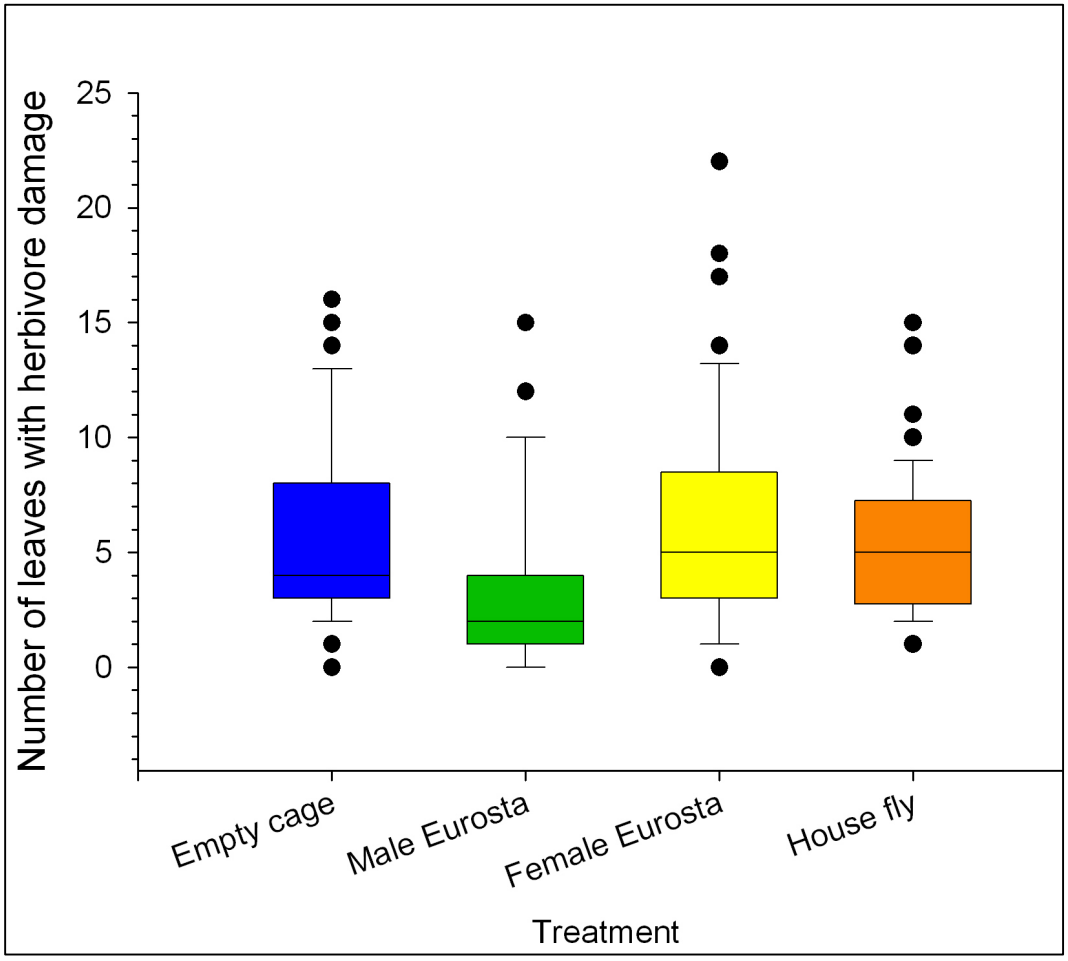

Figure 2-3
Rates of herbivory (number of damaged leaves) on plants exposed to the male *E. solidaginis* emission compared to the three control groups (2 weeks following exposure). The gray box in the figure represents the 25th to 75th percentiles, the line within the box marks the median, the error bars indicate the 10th and 90th percentiles, and the points represent outlying values.

Figure 2-4 A,D
(A) *T. virgata* adult feeding upon *S. altissima* foliage (Photo credit: Ian Grettenberger).
(D) *T. virgata* larva feeding upon *S. altissima* leaves

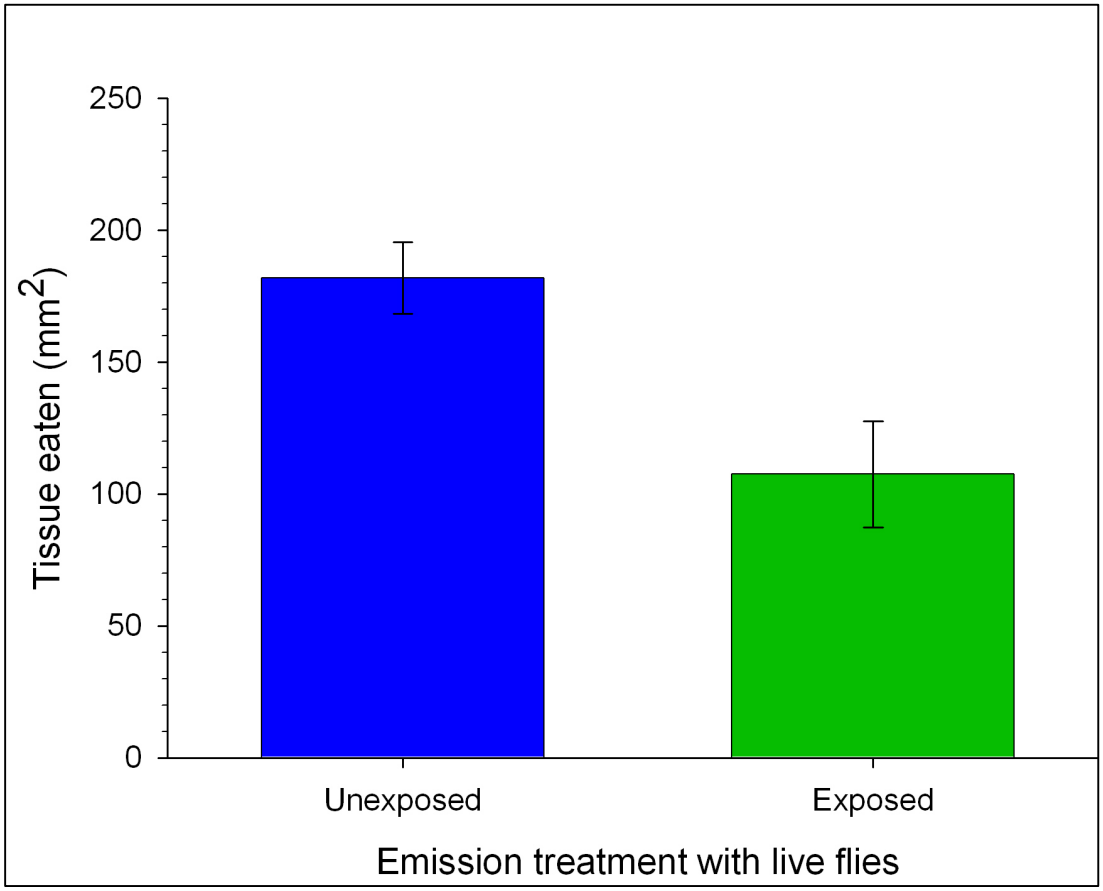

Figure 2-5
Amount of feeding by *T. virgata* adults on *S. altissima* plants exposed to the emission released by adult male *Eurosta solidaginis* or unexposed plants

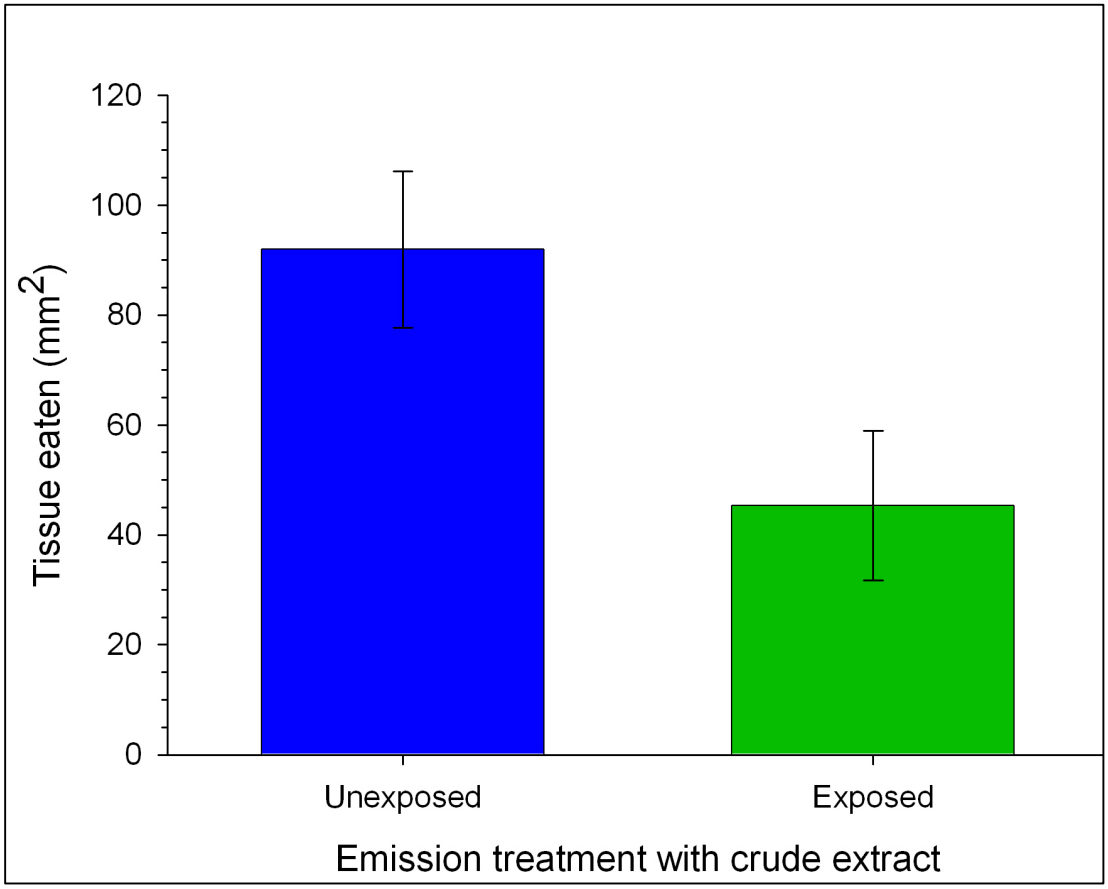

Figure 2-6
Amount of feeding by *T. virgata* adults on *S. altissima* plants exposed to crude extracts of male *Eurosta solidaginis* emission or solvent controls

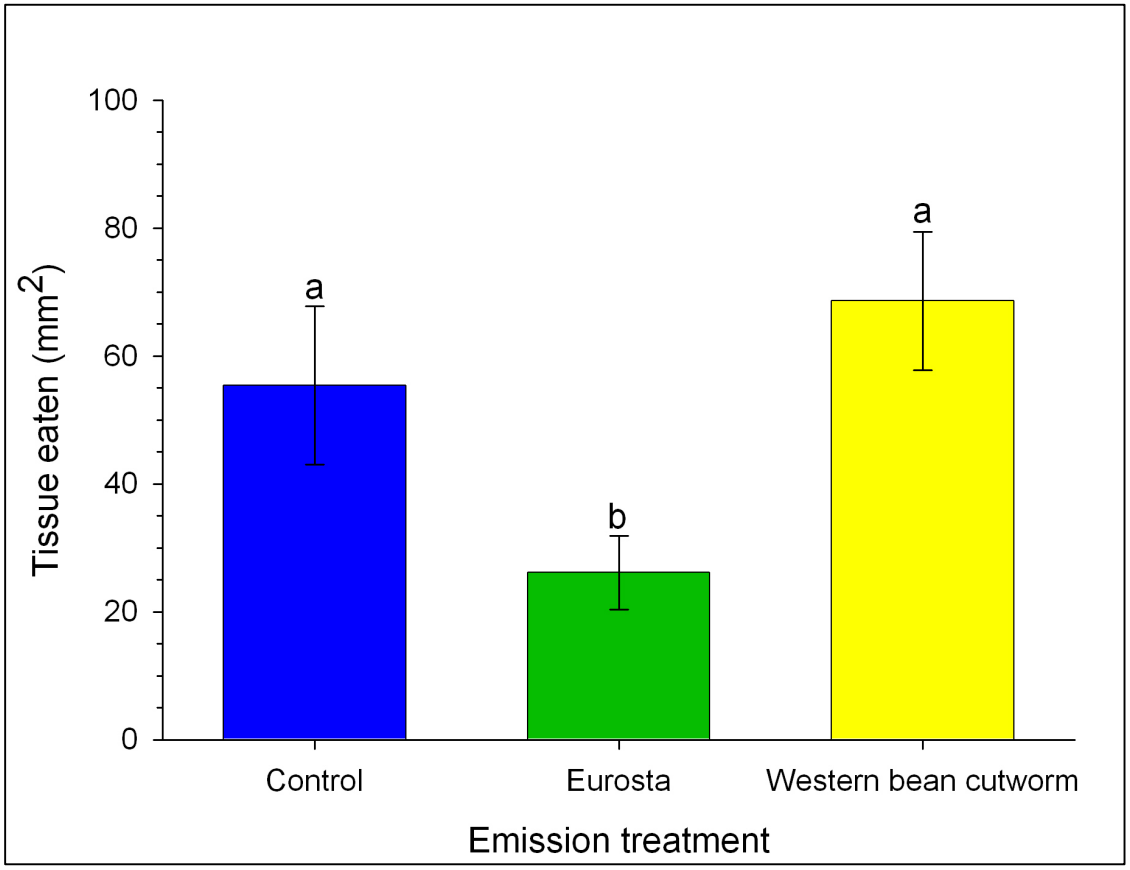

Figure 2-7
Amount of feeding by *T. virgata* larvae on *S. altissima* plants exposed to solvent controls, adult male *E. solidaginis*, or commercially acquired western bean cutworm pheromone (ANOVA $F_{2,23} = 7.8$, $P = 0.003$; data are shown untransformed, but statistics were performed on log-transformed data).

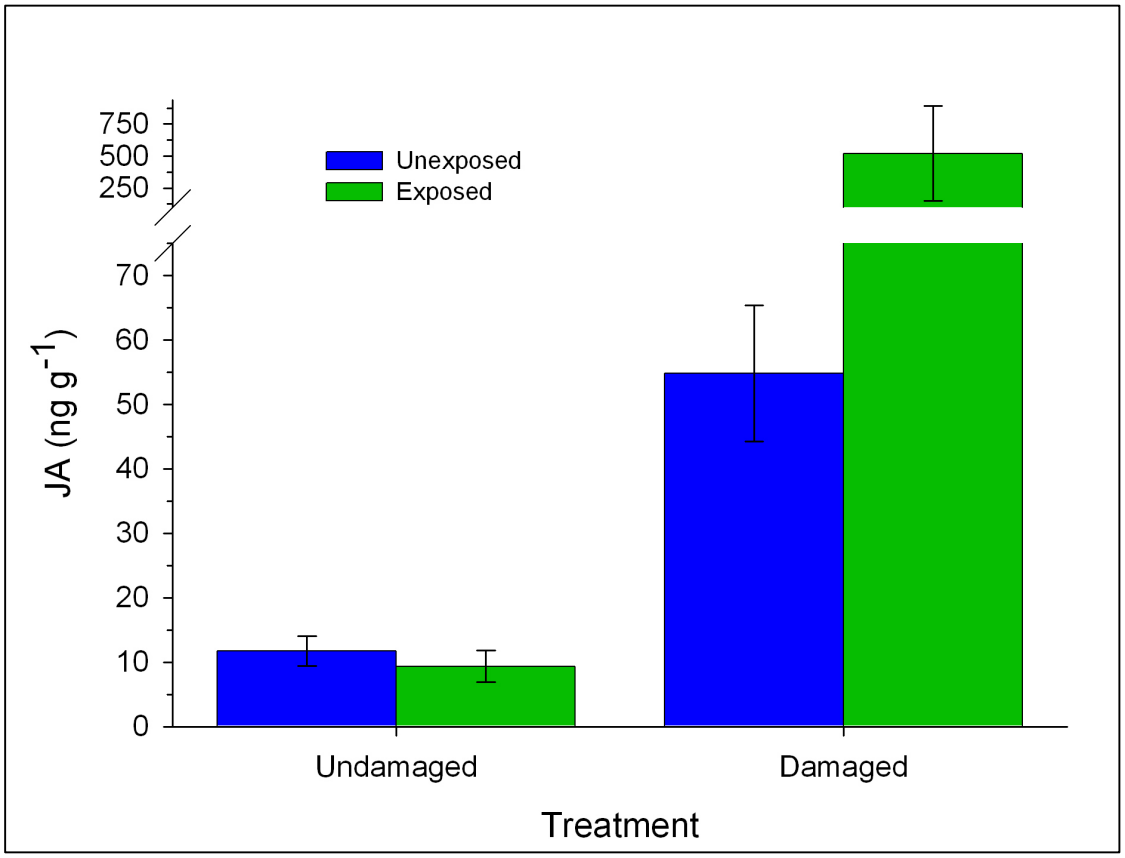

Figure 2-8
Levels of jasmonic acid in *S. altissima* leaves following exposure to the emission of male *Eurosta solidaginis* and herbivory by adult *T. virgata* beetles. After 6 hr of damage, levels of JA were significantly higher when beetles fed upon plants previously exposed to the emission.

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Chapter 3

The volatile emission of *Eurosta solidaginis* primes herbivore-induced volatile production in *Solidago altissima* and does not directly deter insect feeding

Abstract

Background

The induction of plant defenses in response to herbivory is well documented. In addition, many plants prime their anti-herbivore defenses following exposure to environmental cues associated with increased risk of subsequent attack, including induced volatile emissions from herbivore-damaged plant tissues. Recently, I showed in both field and laboratory settings that tall goldenrod plants (*Solidago altissima*) exposed to the putative sex attractant of a specialist gall-inducing fly (*Eurosta solidaginis*) experienced less herbivory than unexposed plants. Furthermore, I observed stronger induction of the defense phytohormone jasmonic acid in exposed plants compared to controls. These findings document a novel class of plant-insect interactions mediated by the direct perception, by plants, of insect-derived olfactory cues. However, my previous

study did not exclude the possibility that the fly emission (or its residue) might also deter insect feeding via direct effects on the herbivores.

Results

Here I show that the *E. solidaginis* emission does not (directly) deter herbivore feeding on *Cucurbita pepo* or *Symphyotrichum lateriflorum* plants—which have no co-evolutionary relationship with *E. solidaginis* and thus are not expected to exhibit priming responses to the fly emission. I also document stronger induction of herbivore-induced plant volatiles (HIPV) in *S. altissima* plants given previous exposure to the fly emission relative to unexposed controls. No similar effect was observed in maize plants (*Zea mays*), which have no co-evolutionary relationship with *E. solidaginis.*

Conclusions

Together with my previous findings, these results provide compelling evidence that reduced herbivory on *S. altissima* plants exposed to the emission of male *E. solidaginis* reflects an evolved plant response to olfactory cues associated with its specialist herbivore and does not involve direct effects of the fly emission on herbivore feeding behavior. I further discuss mechanisms by which the priming of HIPV responses documented here might contribute to enhanced *S. altissima* defense against galling.

Introduction

Despite their sedentary lifestyles, plants actively perceive and respond to a wide range of environmental cues, including those associated with attack by insect herbivores. Induction of plant defenses following insect herbivory is well characterized (1-4). And recent work has shown that, prior to the onset of feeding, many plant species also express or prime anti-herbivore defenses in response to herbivore-associated environmental cues, including both physical and biochemical cues related to the physical presence of herbivores or their eggs (5-7). Defense priming has furthermore been shown to occur in response to airborne chemical cues, specifically damage-induced volatile organic compounds emitted by neighboring plants (or distant parts of the same plant) that are already experiencing herbivory (8-14).

Recently, I documented an apparent example of similar defense priming in goldenrod plants (*Solidago altissima*) exposed to an olfactory cue derived directly from an insect herbivore—the putative sex pheromone of the specialist gall-inducing fly *Eurosta solidaginis* (15). Specifically, I observed dramatically reduced herbivory—in both laboratory and field studies—on plants exposed to the volatile emission of male flies, as well as enhanced induction of the key defense phytohormone jasmonic acid in emission-exposed plants subjected to insect feeding damage. Building upon this work, the current study elucidates additional effects of exposure to the fly emission on *S. altissima* defense responses, as well as the direct effects of the emission itself on insect feeding.

It is well established that plants can respond to airborne chemicals. For example, the diverse and critical functions of the gaseous phytohormone ethylene have been documented and elucidated over many decades (16- 19). And numerous recent studies have elucidated the responsiveness of plants to environmentally derived olfactory cues. Parasitic plants in the genus *Cuscuta*, for example, have been shown to grow toward host-plant-derived volatiles (20), and, as noted above, plants can respond to plant odors elicited by insect feeding (9-13, 21-23).

Our demonstration of *S. altissima* responses to the putative sex attractant of *E. solidaginis* (15) documented a novel class of plant-insect interactions mediated by plant perception of olfactory cues deriving directly from insect antagonists. In that study, I also proposed two alternative hypotheses that might influence the interpretation of my findings: (i) that the effects observed might reflect a biochemical manipulation of the host plant by the fly (rather than an adaptive plant response to a cue indicating the presence of the fly), and (ii) that some residue of the fly emission present on plant tissues might itself deter subsequent herbivory. The first of these hypotheses is difficult to reconcile with my previous finding that female *E. solidaginis* discriminate against emission-exposed plants in the field, which strongly suggests that the quality of these plants as hosts for fly offspring was compromised rather than enhanced (15). The second alternative hypothesis is also countered by my previous findings, specifically the observation of significantly enhanced JA responses of exposed plants to subsequent herbivory, indicating that the observed effects are indeed mediated by physiological responses of the plant to exposure. However, the existence of such enhancement does not exclude the possibility that the fly emission might also have directly deterrent effects on insect feeding that contribute to the

subsequent reduction in herbivory. The current study therefore sought to provide additional evidence that exposure to the *E. solidaginis* emission induces changes in *S. altissima* defense chemistry and to directly test the influence of the emission on feeding by insects.

To further explore *S. altissima* defense responses, I analyzed the volatile production of *S. altissima* plants exposed to the *E. solidaginis* emission and unexposed controls, both before and after herbivore damage. In addition to providing olfactory cues for neighboring plants, as discussed above, herbivore-induced changes in plant volatile emissions are thought to confer defensive benefits by providing cues that recruit natural enemies of feeding herbivores (24-29) or deter feeding or oviposition by additional herbivores (30-33). Furthermore, volatile induction is known to be mediated by JA (34, 35) and thus is likely to reflect downstream influences of the JA induction I documented previously. In addition to examining the effects of the *E. solidaginis* emission on *S. altissima* volatile responses, I conducted parallel experiments in maize (*Z. mays*). Because maize has no apparent co-evolutionary or ecological relationship with *E. solidaginis* and I did not previously observe reduced insect feeding on exposed maize (15), I predicted that exposure to the *E. solidaginis* emission would not induce changes in volatile induction in this plant species.

To explore potential direct effects of the *E. solidaginis* emission on insect feeding, I performed feeding assays using striped cucumber beetles (*Acalymma vittatum*) feeding on *E. solidaginis* emission-exposed squash plants (*Cucurbita pepo* var. *texana*) or on unexposed controls and performed similar assays using goldenrod leaf beetles (*Trirhabda virgata*) feeding on emission-exposed calico aster (*Symphyotrichum lateriflorum*) or

controls. Again because of the absence of any apparent association between *E. solidaginis* flies and squash or calico aster plants I did not expect squash or calico aster to exhibit any physiological response to the *E. solidaginis* emission, so that any reduction in feeding damage observed could likely be attributed to the direct deterrent effect of the emission.

Results

Volatile Collections

To determine whether exposure to the *E. solidaginis* emission primed herbivore-induced volatile production in *S. altissima* plants, I analyzed the volatiles produced by *S. altissima* plants exposed to the emission and unexposed plants both before and after feeding damage by *Heliothis virescens* caterpillars. This generalist caterpillar species was used in place of *E. solidaginis* for the volatile-induction assays because it triggers a strong volatile response from *S. altissima* and because the galling habit of the flies makes them difficult to use for such assays (36). Furthermore, by substituting a generalist leaf-chewing herbivore, I was able to compare volatile-induction by the same herbivore in both maize and goldenrod. Prior to herbivory, I found no difference in volatile production between *S. altissima* plants previously exposed to the emission and unexposed control plants (Additional file 1: Table S1). After feeding by *H. virescens* caterpillars, however, I found that the emission-exposed plants produced a greater total amount of herbivore-induced plant volatiles (HIPV) both during the day (29.0 ng cm^{-2} and 51.8 ng cm^{-2} for unexposed and exposed plants, respectively) and at night (6.2 ng cm^{-2} and 19.1 ng cm^{-2}),

indicating a more vigorous response to insect damage (Fig. 1A, Day: two-sided *t*-Test, $t = -1.93$, df = 18, $P = 0.069$; Fig. 1B, Night: two-sided t-Test, $t = -3.00$, df = 18, $P = 0.0078$). I collected volatiles during both the photophase and scotophase because previous studies documented substantial variation in volatile blends emitted during these phases and day- or night-active insects can be more responsive to the volatiles emitted during their times of peak activity (31, 33).

I also conducted principal component analyses for both the daytime and nighttime HIPV and plotted the first two components from each to visualize which compounds in the blends might be driving the differences between treatments (Additional File 2: Fig. S1A, S1B). For daytime HIPV, the first two principal components account for 96.3% of the variance. For nighttime HIPV, the first two principal components account for 96.4% of the variance. In total, I measured and identified twenty-three compounds in the *S. altissima* volatile blend. I found no novel compounds in the HIPV blend of emission-exposed plants compared to the control or when comparing the blends of damaged and undamaged plants; however, I identified a few specific compounds in the daytime and nighttime blends that were emitted in significantly greater amounts by the induced emission-exposed plants (Table 1). The compounds emitted in significantly greater amounts ($P \leq 0.05$) in the daytime blend were the monoterpenes α-pinene, β-pinene, and limonene. Three compounds were also marginally significant, including bornyl acetate ($P = 0.09$) and the monoterpenes camphene ($P = 0.09$) and myrcene ($P = 0.06$). In the nighttime blend, emission-exposed *S. altissima* emitted the following compounds in significantly higher amounts ($P \leq 0.05$) after herbivore damage: the monoterpenes α-pinene, β-pinene, myrcene, and limonene, the sesquiterpenes caryophyllene, α-humulene,

β-farnescene, and germacrene D, and the compound bornyl acetate. Four compounds were marginally significant, including the green-leaf volatiles (GLV) (*Z*)-3-hexen-1-ol ($P = 0.07$) and (*Z*)-3-hexenyl isobutyrate ($P = 0.07$), the terpene alcohol linalool ($P = 0.09$) and (*Z*)-jasmone ($P = 0.06$). No compounds were emitted in significantly higher amounts by the unexposed control plants.

To test whether this observed increase in HIPV production following exposure to the *E. solidaginis* emission represented a specific response from the co-evolved host plant species or a general plant response to the compounds in the fly emission, I also examined the influence of exposure to the *E. solidaginis* emission on volatile production in maize plants (*Z. mays*). As for *S. altissima*, I found no difference between the total volatile production from undamaged maize plants exposed to the emission or undamaged controls; however, here I also found no difference between the volatile blends induced by *H. virescens* feeding damage on exposed or control plants, indicating that the maize plants did not respond to the *E. solidaginis* emission by enhancing HIPV production (Additional file 3: Table S2). Herbivore-damaged maize plants exposed to the *Eurosta* emission produced 89.2 ± 80.3 ng cm^{-2} during the day and unexposed controls produced 143.9 ± 42.9 ng cm^{-2}. Nighttime HIPV production from *Eurosta*-exposed plants was 21.2 ± 8.3 ng cm^{-2} and 13.1 ± 6.6 ng cm^{-2} from unexposed control plants. (Day: two-sided *t*-Test, $t = 0.60$, df = 14, $P = 0.56$; Night: two-sided *t*-Test, $t = -0.77$, df = 14, $P = 0.46$).

Feeding Assays

To test the hypothesis that the presence of the *E. solidaginis* emission might directly deter insect feeding on exposed plants, I conducted feeding assays with the

specialist beetle herbivore *A. vittatum* feeding on exposed and unexposed *C. pepo* plants. *Cucurbita pepo* is only distantly related to *S. altissima* and has no apparent association with *E. solidaginis*. I also performed a similar feeding assay with *T. virgata* feeding on exposed and unexposed *Symphyotrichum lateriflorum. Trirhabda virgata* was employed in my previous study of plant responses to the *E. solidaginis* emission and was found to consume less leaf tissue on emission-exposed *S. altissima* (15). This species naturally feeds upon *Solidago* and a few closely related genera, including *Symphyotrichum* (37-39). *Symphyotrichum lateriflorum* is a suitable host plant species for *T. virgata* but not for *E. solidaginis;* therefore, I predicted that exposure to the *E. solidaginis* emission would not enhance *Symphyotrichum lateriflorum* defenses or deter *T. virgata* feeding. I found no significant difference in the total amount of leaf tissue consumed by *A. vittatum* beetles on exposed or control *C. pepo* (Fig. 2A, two-sided *t*-test, t_{18}=0.41, P =0.69) or *T. virgata* feeding on exposed or control *Symphyotrichum lateriflorum* (Fig. 2B, two-sided *t*-test, t_{12}=0.16, P =0.87), suggesting that these plant species, which again do not appear to have co-evolutionary history with *E. solidaginis*, did not alter their defenses in response to its emission and the emission did not directly deter herbivore feeding.

Discussion

I previously reported that *S. altissima* plants perceive the volatile emission produced by male *E. solidaginis* flies and respond by enhancing their anti-herbivore defenses (15). The findings presented here provide additional physiological evidence of this phenomenon and reveal that a specific downstream anti-herbivore defense—

herbivore-induced volatile production—is primed by exposure to the emission. Although the ecological significance of HIPV in this system has not been explored, it is likely that *S. altissima* volatile emissions play a role in plant defense against insect herbivores (36), as has been found for numerous plant species (24-33, 40).

Exposure to the emission of male *E. solidaginis* enhanced daytime herbivore-induced volatile production by roughly 44% and nighttime production by roughly 68%. These substantial differences in HIPV production would appear to provide a strong signal for members of the associated arthropod community, possibly including foraging predators and parasitoids. *Eurytoma* parasitoids of *E. solidaginis* are active during the day (personal observation, Abrahamson and Weis 1997), but I am unaware of any efforts to characterize their activity levels at night. Given the considerably stronger induction of nighttime volatiles, it seems reasonable to hypothesize that night-active natural enemies may be able to exploit these cues.

A previous study found that primed maize plants, which released more concentrated HIPV emissions preferentially attracted natural enemies compared to unprimed control plants (41). Some of the compounds emitted in higher concentrations by damaged, *E. solidaginis*-emission-exposed *S. altissima* have previously been linked to defensive roles against insect herbivores in other systems. Green-leaf volatiles (GLV) and terpenes, for example, provide important signals for parasitoid and predator attraction, herbivore repellence, and reduced herbivore performance (30,31, 40, 42-44). Additionally, some of the sesquiterpenes primed in this study, including β-farnescene, β-caryophyllene and germacrene D, were also emitted in higher quantities by primed poplar trees and/or maize plants exposed to HIPV (11, 45).

In contrast to the *S. altissima* HIPV response following *H. virescens* attack, I observed no similar increase in HIPV production when the same generalist caterpillar species attacked emission-exposed maize plants. These contrasting results support my hypothesis that *S. altissima* plants exhibit an evolved ability to perceive and respond to the emission of its closely associated herbivore *E. solidaginis*. It seems likely that other plant species may also have evolved the ability to detect the pheromones of their herbivores, but I hypothesize that this adaption is most likely to have developed in closely co-evolved plant-insect interactions, likely with monophagous or narrowly oligophagous herbivore species that have a strong influence on host-plant fitness (15).

In my previous work, I observed that both larvae and adults of *T. virgata* consumed less leaf tissue from *S. altissima* plants exposed to the *E. solidaginis* emission compared to control plants (15). In the same study, I also observed a general reduction of herbivory on the emission-exposed plants in my field experiment, these results are consistent with my hypothesis that the reduced feeding on emission-exposed *S. altissima* plants was the result of an evolved response by *S. altissima* to its specialist herbivore *E. solidaginis*. In the current study, I observed no difference in the feeding of *A. vittatum* on their preferred host plant species *C. pepo,* (which like maize has no obvious relationship to *E. solidaginis*) with and without exposure to the volatile emission of the fly. I also found no difference in feeding damage by *T. virgata* on emission-exposed or control *S. lateriflorum.* This latter result is of particular interest because *Symphyotrichum lateriflorum* is a close relative of *S. altissima* that is a suitable host plant for *T. virgata* but not *E. solidaginis* (37-39). These results thus strongly indicate that insect herbivores, in

this case two herbivorous chrysomelid species, are not directly deterred by the *E. solidaginis* emission.

Conclusion

The findings presented here provide further support for my hypothesis that *S. altissima* plants can perceive and respond to the putative sex attractant of *E. solidaginis*. In contrast, I found no evidence that the *E. solidaginis* emission directly deters insect feeding. Furthermore, the enhancement of HIPV induction in emission-exposed *S. altissima* plants observed here complements my previous finding that JA induction by herbivory is enhanced in *S. altissima* plants given prior exposure to the fly emission (15), providing additional evidence that reduced herbivory on *S. altissima* plants following exposure to the volatile emission of *E. solidaginis* indeed reflects an evolved adaptive response of this plant species to an olfactory cue from its closely associated herbivore. I can therefore conclude with greater certainty that this system provides the first example of a novel class of plant-insect interactions mediated by plant perception of insect-derived olfactory cues.

Methods and Materials

The study system

Adult *E. solidaginis* flies typically emerge in mid-May in Pennsylvania and male flies seek perches on goldenrod plants from which to attract mates (46, 47). I discovered

that while perching on plants, the male flies emit large quantities of a putative sex pheromone, attractive to female flies (mean ~70 $\pm$ 20 μg 24 h^{-1}; [15]). After mating, females begin searching for suitable oviposition sites, often ovipositing into the stem of the same or nearby plants. Reproductive output of *S. altissima* plants suffers significantly from galling by *E. solidaginis* (47); thus, detecting reliable cues associated with impending attack, such as the male fly emission, could provide plants with an advantage in their defense against *E. solidaginis* attack (15). *E. solidaginis* eggs hatch within 5-8 days and the larval-induced galls usually become visible within 3 weeks (47).

Plants

I propagated tall goldenrod (*Solidago altissima*) plants from rhizomes of the 110 clone line and grew them in insect-free, climate-controlled growth chambers (16 h light: 8 h dark; 22 °C: 20 °C; 65% relative humidity (RH)). Rhizomes for this experiment were grown from *S. altissima* originally collected from a field near State College, PA, USA and washed and stored at 4 °C prior to planting. I cut rhizomes of similar diameter into 5 cm segments and planted them in shallow trays with peat-based potting soil (Pro-Mix BX; Premier Horticulture Inc., Quakertown, PA, USA). Two weeks after planting, I transplanted the sprouted ramets into individual pots (16 cm diameter, 16.5 cm tall) using the same type of soil and added 0.5 tsp Osmocote fertilizer (8–45–14 N–P–K, Scotts, Marysville, OH, USA) to each pot. *S. altissima* plants used in experiments were 8 wk old and ~ 35 cm tall.

I grew maize plants (*Zea mays* cv. Delprim) from seed in insect-free, climate-controlled growth chambers (16 h light: 8 h dark; 25 °C: 25 °C; 65% RH). I germinated

seeds in the peat-based potting soil and transplanted seedlings into individual pots approximately 1 wk after germination. At this time, plants received 0.5 tsp of the Osmocote fertilizer. *Z. mays* plants used in experiments were in the 3 leaf stage.

I grew wild gourd (*Cucurbita pepo* var. *texana*) plants from seed in insect-free, climate-controlled growth chambers (16 h light: 8 h dark; 23 °C: 21 °C; 65% RH). I planted seeds in the peat-based potting soil with 0.5 tsp Osmocote fertilizer. *C. pepo* plants used in this experiment were 3.5 weeks old (4 fully expanded leaves).

I grew calico aster (*Symphyotrichum lateriflorum*) plants from rhizomes in insect-free, climate-controlled growth chambers (16 h light: 8 h dark; 23 °C: 21 °C; 65% RH). The rhizomes for this experiment were harvested from plants grown from seed (Prairie Moon Nursery, Winona, MN, USA) under these same conditions. Importantly, this seed source is within the natural range *E. solidaginis* and its *Solidago* host plant species (47). The rhizomes were harvested, washed and stored at 4 °C prior to planting. I planted 2 cm segments of rhizome in the peat-based potting soil with 0.5 tsp Osmocote fertilizer. *S. lateriflorum* plants used in this experiment were 4 weeks old with a basal rosette of leaves and ~ 20 cm stalk.

Insects

I collected adult male *Eurosta solidaginis* after they emerged from overwintering galls that I had collected near State College, PA, USA and stored at -20 °C. To induce emergence, I placed the galls in a climate-controlled incubator (16 h light: 8 h dark; 22 °C, 20 °C; 65% RH) for approximately 3 wk.

I reared tobacco budworm (*Heliothis virescens*) larvae in a climate-controlled incubator (16 h light: 8 h dark; 22 °C, 20 °C; 65% RH) from purchased eggs (Bio-Serv, Frenchtown, NJ, USA) and fed them an artificial casein-based diet. *H. virescens* used in experiments were fourth-instar larvae and were starved for 24 h at room temperature prior to the experiments. Feeding by *H. virescens* caterpillars was previously found to elicit strong volatile production in *S. altissima* plants (36).

I reared striped cucumber beetles (*Acalymma vittatum*) in a laboratory colony from adults collected near State College, PA, USA and fed them growth-chamber grown cucumber plants. Cucumber beetles used in the experiment were mature adults and were starved for 24 h at room temperature prior to the experiment.

I collected goldenrod leaf beetles (*Trirhabda virgata*) from a natural population near State College, PA, USA. I fed the beetles growth-chamber grown *S. altissima* and then starved them for 24 h at room temperature prior to the experiment. Each plant in the experiment received two adult female and one adult male *T. virgata* beetles.

Collection of the *E solidaginis* Emission

Following my previously described methods, I collected the male *E. solidaginis* emission by aerating newly emerged adult male flies in small glass chambers for 24 h (15). I pushed filtered house air into the chambers at 0.6 $L \cdot min^{-1}$ and pulled air out of the chambers, over an adsorbent filter containing 45 mg of Super-Q (Alltech Associates, Deerfield, IL, USA) at 0.5 $L \cdot min^{-1}$. I eluted filters using 150 μL of dichloromethane and individual samples were pooled to ensure a uniform concentration of emission for the exposure treatments.

Emission Exposure Treatments

Inside individual glass chambers (4-L volume), I exposed *S. altissima, Z. mays, C. pepo,* and *S. lateriflorum* plants to crude extracts of the male *E. solidaginis* emission or a dichloromethane solvent control for 24 h (15). Chambers rested on a two-piece aluminum and Teflon base supported by the rim of the plant pots. The stem of the plant passed through a hole in the aluminum base and was wrapped with cotton to fill the space between the stem and base. To prevent accumulation of condensation and an unrealistic concentration of the *E. solidaginis* emission from building up, filtered air was pushed into the chambers at 3.0 $L \cdot min^{-1}$ and pulled out at 1.0 $L \cdot min^{-1}$. I allowed plants to acclimate to the chambers for 1 h before beginning the exposure treatment. I applied a 12-h male equivalent (40 μL) of the *E. solidaginis* emission crude extract or dichloromethane to each rubber septa and added two septa to each glass chamber. After 12 hours, I added two fresh emission- or solvent-containing septa to each chamber.

Volatile Collections

Using an automated push-pull volatile collection system (Analytical Research Systems, Gainsville, FL, USA), I collected plant-produced volatile compounds from exposed *S. altissima* and *Z. mays* plants before and after herbivore damage. Volatile collections were conducted in a climate-controlled growth chamber (16 h light: 8 h dark; 22 °C, 20 °C; 65% RH). During the collection, filtered air was delivered into each chamber at 3.0 $L \cdot min^{-1}$ and pulled out of the chamber through an adsorbent filter (containing 45 mg of Super-Q [Alltech Associates, Deerfield, IL, USA]) at 1.0 $L \cdot min^{-1}$. I

collected volatiles for 16 h during photophase (06:00-22:00) and on a separate set of filters for 8 h during scotophase (22:00-06:00). After collecting from undamaged plants for 24 h, I introduced two 4th instar *H. virescens* caterpillars into each chamber and allowed them to feed on the plants for 24 h. During this time, I collected damage-induced volatiles following the same schedule. After 24 h, I removed the insects, harvested the plants, and scanned the leaves to calculate the leaf area.

I eluted the volatile trap filters using 150 µL dichloromethane and added to each sample 5 µL of a standard containing nonyl acetate (80 ng/µL) and *n*-octane (40 ng/µL). I quantified amounts of compounds in samples using an Agilent model 7890A gas chromatograph fitted with a flame ionization detector, using a splitless injector held at 220˚C. The column (HP-5, 15 m x 0.25 mm x 0.25 µm film thickness; J&W Scientific, Folsom, CA) was maintained at 35 ˚C for 30 s, then ramped 2˚C min^{-1} to 130˚C, and ramped again at 20˚C min^{-1} to 220˚C. I identified volatile components with gas chromatography (Agilent model 7890A) coupled with a mass spectrometer (Agilent model 5975C) in electron ionization mode comparing retention times and spectra with that of pure compounds. Following quantification, the volatile production for each plant was corrected by the total leaf area for that plant (ng cm^{-2}). I corrected the volatile production (ng) by the total leaf area (cm^2) to account for size variation among plants that might have influenced volatile production. To obtain the leaf area, I destructively sampled plants immediately following the collections. Consequently, I used the same leaf-area value to correct the day and night volatiles (neglecting limited leaf area growth during the collection periods).

Feeding Assays

I conducted insect herbivore feeding assays using *C. pepo* and *S. lateriflorum* exposed to either the *E. solidaginis* emission or a solvent control. I exposed plants to a crude extract of the emission or a dichloromethane solvent control following the procedure described above. After 24 h of exposure, I introduced three *A. vittatum* to each of the *C. pepo* and three *T. virgata* to each of the *Symphyotrichum lateriflorum* allowed them to feed on the plants. After 24 h of feeding, I harvested the plants and the scanned their leaves to determine the total area of leaf tissue consumed.

Statistical Analyses

I analyzed the plant volatile data by calculating the herbivore damage-induced volatiles (ng cm^{-2}) produced by each plant (herbivore-damaged plant volatiles – undamaged plant volatiles) during a given time period. I calculated the induced value for each compound in the volatile blend and summed the values to obtain the total induced volatiles for each plant. To account for potential differences in volatile production due to plant size differences, I corrected the induced volatile values for the total leaf area (cm^2) of the plant. I transformed the *S. altissima* volatiles using a square-root transformation to meet the assumptions of normality and equal variance. I then compared the herbivore-induced volatiles from the emission-exposed and unexposed plants using a two-sided *t*-test for both *S. altissima* and *Z. mays*. I conducted a principle component analysis for the individual compounds of both the day and night *S. altissima* HIPV and constructed biplots of the results. Based on these biplots as well as the standard errors for each

compound, I selected individual compounds to test using pair-wise comparisons. A two-sided *t*-test was used to compare the amount of leaf tissue consumed in the *C. pepo* and *Symphyotrichum lateriflorum* feeding assays.

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Figures and Tables

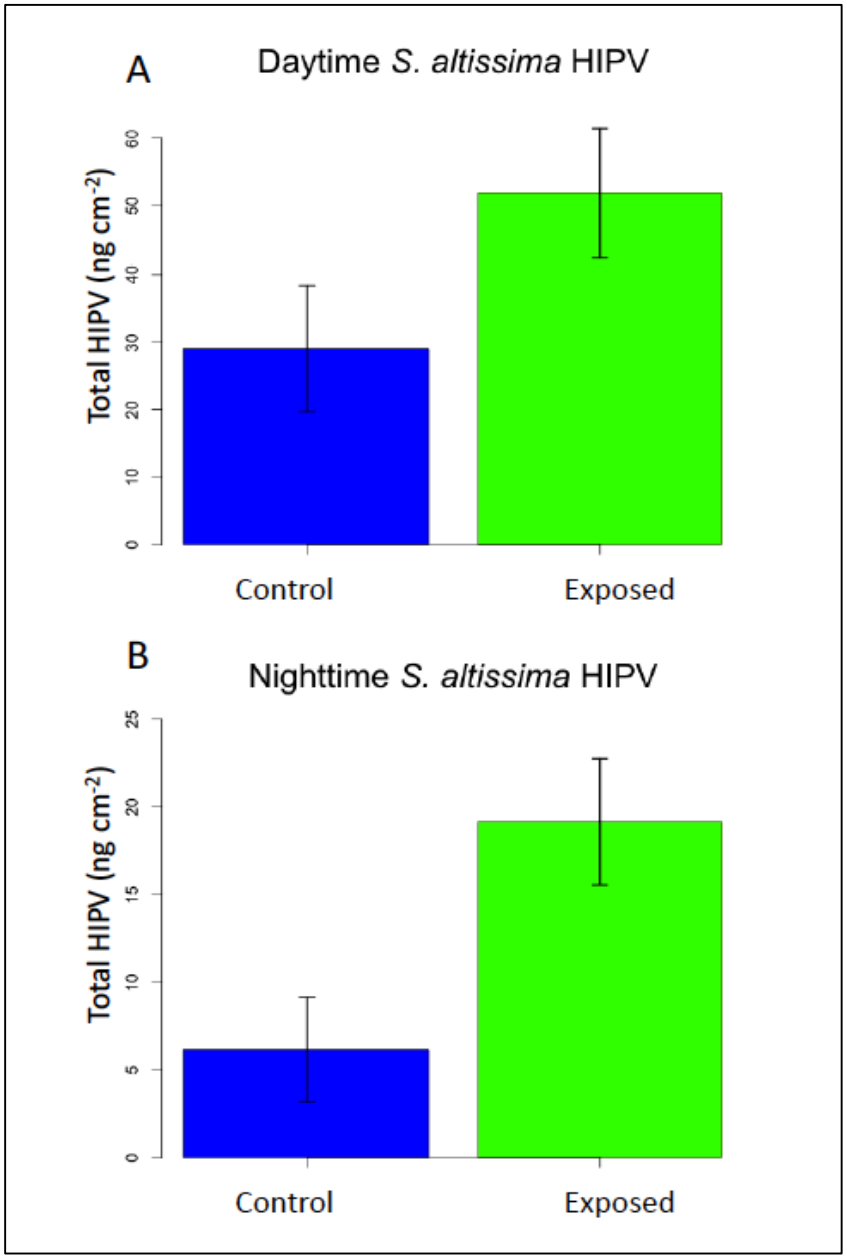

Figure 3-1
Solidago altissima herbivore-induced volatiles. (A) Total herbivore-induced volatiles emitted by *S. altissima* plants exposed to the *E. solidaginis* emission and unexposed controls during 18 h photophase. (B) Total herbivore-induced volatiles emitted by *S. altissima* plants exposed to the *E. solidaginis* emission and unexposed controls during 6 h scotophase. Data are shown untransformed, but statistical analyses were performed on square-root transformed data.

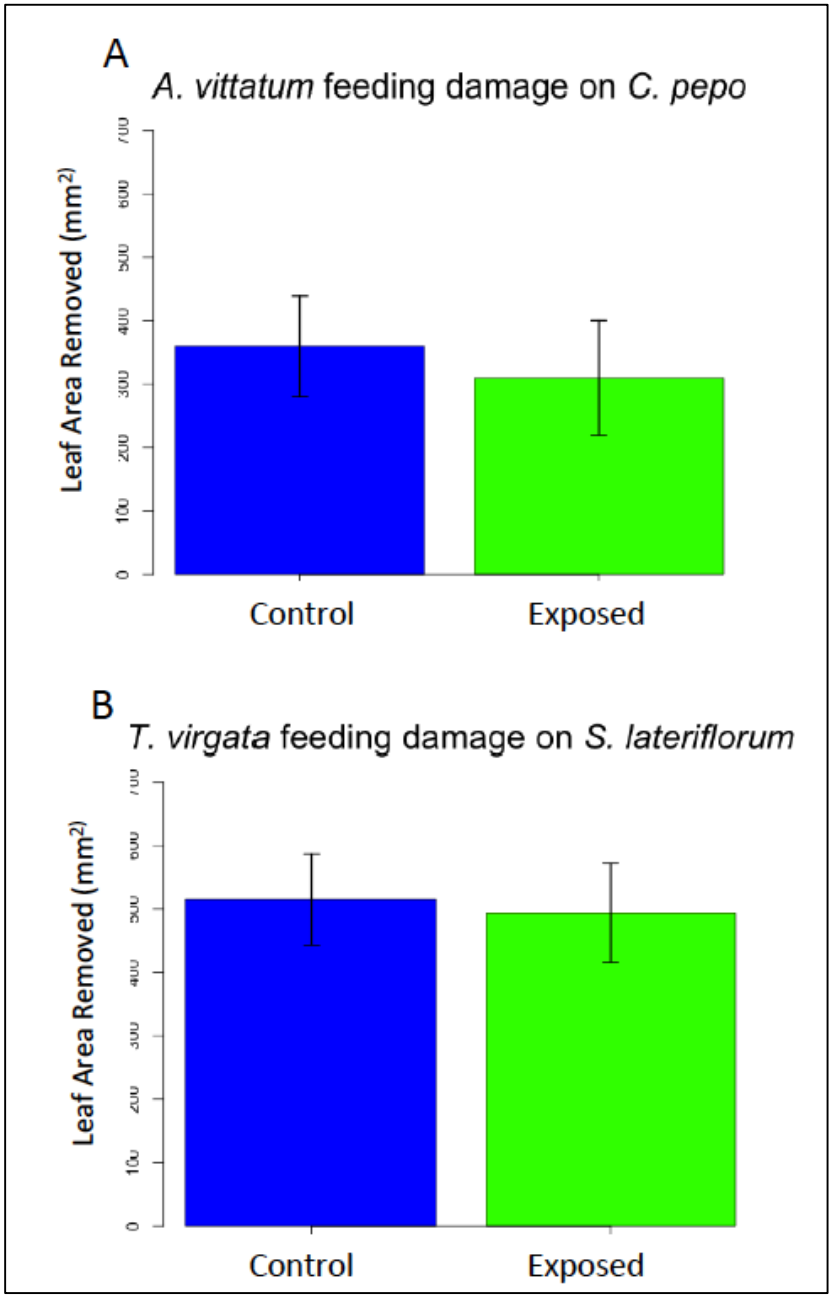

Figure 3-2 A,B

(A) *Acalymma vittatum* feeding damage on *Cucurbita pepo.* Amount of leaf tissue removed by *A. vittatum* feeding on *C. pepo* exposed to the *E. solidaginis* emission or unexposed controls. (B) *Trirhabda virgata* feeding damage on *Symphyotrichum lateriflorum.* Amount of leaf tissue removed by *T. virgata* feeding on *S. lateriflorum* exposed to the *E. solidaginis* emission or unexposed controls.

Compounds in *S. altissima* VOC blend	Daytime HIPV			Nighttime HIPV		
	Exposure treatment					
	Eurosta	Control		*Eurosta*	Control	
	Induced VOC (ng cm^{-2}) ± SE	Induced VOC (ng cm^{-2}) ± SE	*t*-statistic (*P*-value)	Induced VOC (ng cm^{-2}) ± SE	Induced VOC (ng cm^{-2}) ± SE	*t*-statistic (*P*-value)
(*Z*)-3-hexen1ol	1.00 ± 0.03	0.32 ± 0.17	1.62 (0.12)	0.84 ± 0.22	0.04 ± 0.35	1.95 (0.07)
α-pinene	9.29 ± 2.8	2.79 ± 0.95	2.83 (0.01)*	3.76 ± 0.93	1.49 ± 0.62	2.76 (0.01)*
Camphene	0.56 ± 0.27	0.13 ± 0.08	1.78 (0.09)	0.11 ± 0.04	0.06 ± 0.04	-
β-pinene	4.45 ± 1.4	1.44 ± 0.49	2.73 (0.01)*	1.12 ± 0.36	0.44 ± 0.21	2.56 (0.02)*
Myrcene	7.42 ± 1.3	5.43 ± 1.7	2.03 (0.06)	1.68 ± 0.31	0.69 ± 0.24	2.88 (0.01)*
(*Z*)-3-Hexenyl acetate	5.43 ± 1.8	3.41 ± 1.4	-	5.53 ± 0.83	0.92 ± 0.83	1.63 (0.12)
Limonene	14.3 ± 2.9	8.76 ± 3.2	2.12 (0.05)*	3.31 ± 0.54	1.53 ± 0.54	2.65 (0.02)*
(*E*)-β-ocimene	0.66 ± 0.25	0.39 ± 0.33	-	0.11 ± 0.04	0.05 ± 0.04	-
Linalool	0.22 ± 0.05	0.20 ± 0.094	-	0.09 ± 0.02	0.05 ± 0.02	1.81 (0.09)
Nonatriene[1]	0.93 ± 0.27	0.98 ± 0.46	-	0.16 ± 0.05	0.11 ± 0.05	-
(*Z*)-3-hexenyl isobutyrate	0.07 ± 0.04	0.03 ± 0.018	-	0.09 ± 0.01	0.03 ± 0.01	1.93 (0.07)
(*Z*)-3-hexenyl butyrate	0.07 ± 0.02	0.062 ± 0.023	-	0.03 ± 0.01	0.04 ± 0.01	-
(*E*)-2-hexenyl butyrate	0.09 ± 0.02	0.089 ± 0.036	-	0.04 ± 0.01	0.03 ± 0.01	-
Bornyl acetate	0.89 ± 0.32	0.594 ± 0.33	1.74 (0.09)	0.03 ± 0.01	0.12 ± 0.06	2.2 (0.04)*
(*Z*)-jasmone	0.27 ± 0.14	0.224 ± 0.093	-	0.04 ± 0.03	-0.07 ± 0.03	2.1 (0.04)*
β-caryophyllene	0.84 ± 0.23	0.655 ± 0.24	-	0.28 ± 0.05	0.12 ± 0.05	2.1 (0.05)*
α-humulene	0.05 ± 0.02	0.006 ± 0.038	-	0.03 ± 0.01	-0.03 ± 0.01	2.9 (0.009)*
β-farnescene	0.27 ± 0.07	0.238 ± 0.084	-	0.09 ± 0.01	0.03 ± 0.01	2.6 (0.02)*
GermacreneD	4.65 ± 1.0	3.37 ± 1.1	-	1.35 ± 0.20	0.46 ± 0.20	2.4 (0.03)*
α-farnescene	0.26 ± 0.09	0.349 ± 0.17	-	0.10 +/ 0.02	0.06 ± 0.03	-
Nerolidol	0.03 ± 0.02	0.10 ± 0.083	-	0.01 ± 0.01	0.01 ± 0.01	-
Tridecatetraene	0.02 ± 0.02	0.05 ± 0.045	-	0.008 ± 0.01	0.02 ± 0.01	-
Indole	0.05 ± 0.02	0.05 ± 0.03	-	0.04 ± 0.02	0.008 ± 0.004	-

Table 1. Day and night *Solidago altissima* individual herbivore-induced volatile organic compounds (VOC**;** means ± standard error; untransformed data shown**)**. Within daytime and nighttime collections, asterisks (*) indicate statistical comparisons (*t*-tests) of volatile constituents that were significantly different ($P \leq 0.05$; data shown are untransformed, but statistical analyses were performed on square-root transformed data) between plants that were exposed to the *E. solidaginis* emission and untreated control plants. Dashes (-) indicate overlapping standard errors so no t-tests were conducted. All compounds detected and identified in the *S. altissima* volatile blend are included here.
[1](3*E*)-4,8-dimethyl-1,3,7-nonatriene

	Daytime VOC		Nighttime VOC	
	Exposure treatment			
	Eurosta	Control	*Eurosta*	Control
S. altissima Volatile Compounds	Undamaged VOC (ng cm^{-2}) ± SE	Undamaged VOC (ng cm^{-2}) ± SE	Undamaged VOC (ng cm^{-2}) ± SE	Undamaged VOC (ng cm^{-2}) ± SE
(*Z*)-3-hexen1ol	0.234 ± 0.12	0.147 ± 0.09	0.676 ± 0.29	0.780 ± 0.27
α-pinene	0.231 ± 0.09	0.705 ± 0.31	0.000 ± 0.00	0.027 ± 0.02
Camphene	0.090 ± 0.05	0.142 ± 0.08	0.093 ± 0.05	0.059 ± 0.02
β-pinene	0.098 ± 0.04	0.317 ± 0.13	0.000 ± 0.00	0.006 ± 0.01
Myrcene	0.177 ± 0.09	0.417± 0.16	0.000 ± 0.00	0.009 ± 0.01
(*Z*)-3-Hexenyl acetate	0.165 ± 0.05	0.243 ± 0.09	1.814 ± 0.81	2.282 ± 0.74
Limonene	0.458 ± 0.23	1.091 ± 0.40	0.000 ± 0.00	0.024 ± 0.02
(*E*)-β-ocimene	0.467 ± 0.09	0.440 ± 0.13	0.180 ± 0.06	0.115 ± 0.04
Linalool	0.048 ± 0.02	0.046 ± 0.02	0.004 ± 0.00	0.000 ± 0.00
Nonatriene	0.222 ± 0.08	0.533 ± 0.27	0.004 ± 0.00	0.000 ± 0.00
(*Z*)-3-hexenyl isobutyrate	0.030 ± 0.01	0.017 ± 0.02	0.000 ± 0.00	0.000 ± 0.00
(*Z*)-3-hexenyl butyrate	0.014 ± 0.01	0.023 ± 0.01	0.000 ± 0.00	0.006 ± 0.01
(*E*)-2-hexenyl butyrate	0.028 ± 0.01	0.030 ± 0.01	0.000 ± 0.00	0.006 ± 0.01
Bornyl acetate	0.011 ± 0.01	0.098 ± 0.04	0.000 ± 0.00	0.009 ± 0.01
(*Z*)-jasmone	0.055 ± 0.03	0.087 ± 0.03	0.055 ± 0.02	0.117 ± 0.03
Caryophyllene	0.041 ± 0.02	0.076 ± 0.04	0.000 ± 0.00	0.000 ± 0.00
α-humulene	0.110 ± 0.06	0.089 ± 0.03	0.011 ± 0.01	0.036 ± 0.01
β-farnescene	0.047 ± 0.02	0.050 ± 0.02	0.000 ± 0.00	0.000 ± 0.00
GermacreneD	0.095 ± 0.03	0.262 ± 0.17	0.055 ± 0.02	0.104 ± 0.02
α-farnescene	0.052 ± 0.02	0.117 ± 0.04	0.000 ± 0.00	0.000 ± 0.00
Nerolidol	0.025 ± 0.01	0.053 ± 0.03	0.000 ± 0.00	0.000 ± 0.00
Tridecatetraene	0.051 ± 0.03	0.063 ± 0.03	0.000 ± 0.00	0.000 ± 0.00
Indole	0.012 ± 0.01	0.017 ± 0.01	0.000 ± 0.00	0.000 ± 0.00

Table S1.

Volatile organic compounds emitted by undamaged *Solidago altissima* plants. Table showing the individual compounds that make up the volatile blend of undamaged *S. altissima* plants. (VOC; means ± standard error; untransformed data shown).

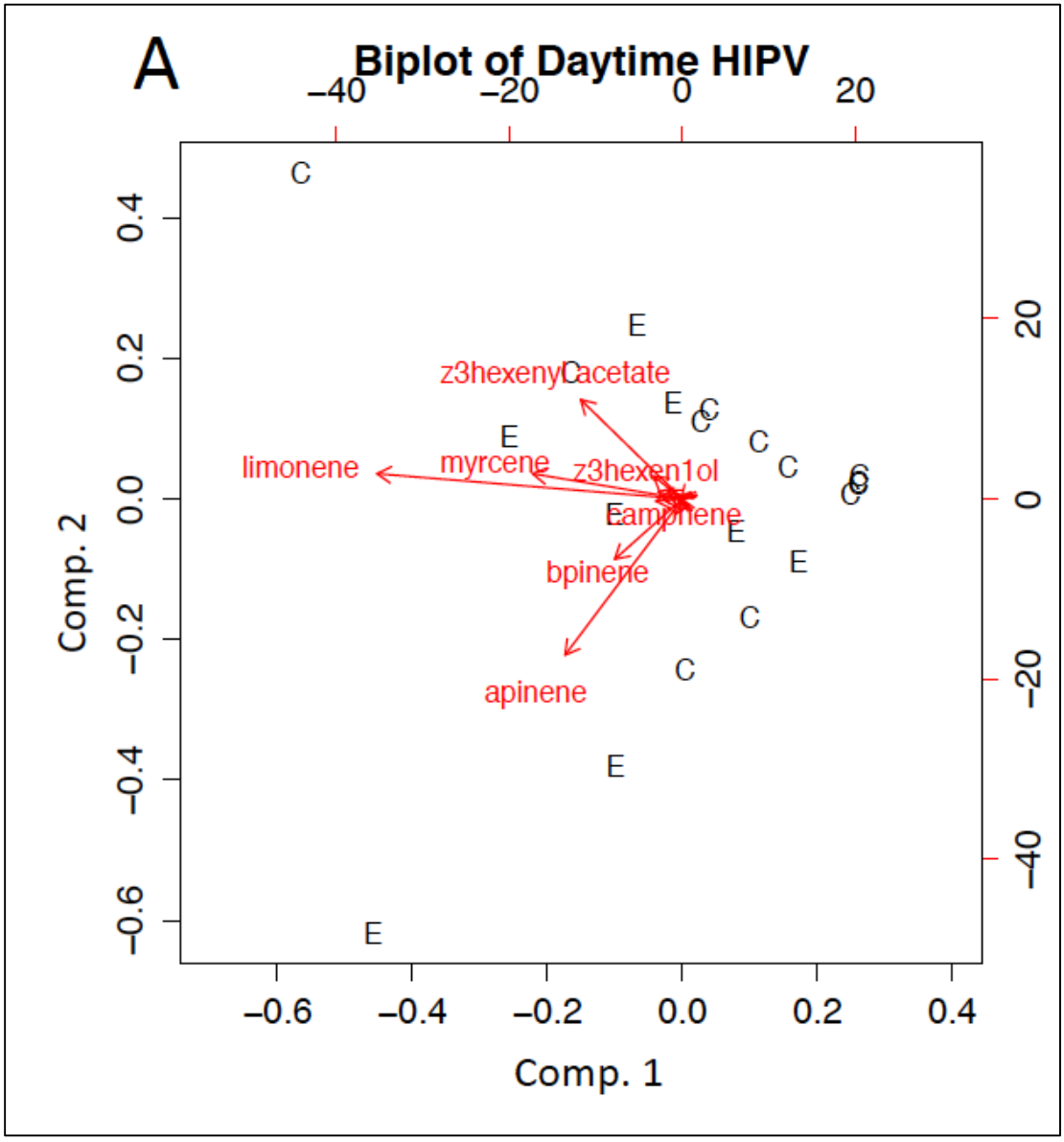

Figure 3-3
Biplot of first two principle components of the photophase herbivore-induced volatiles for *E. solidaginis* emission-exposed and unexposed *S. altissima.* Arrows indicate the weight given to individual compounds. Not all compound labels are shown for legibility. Individual plants are labeled with a character representing the treatment (C = control, E = exposed).

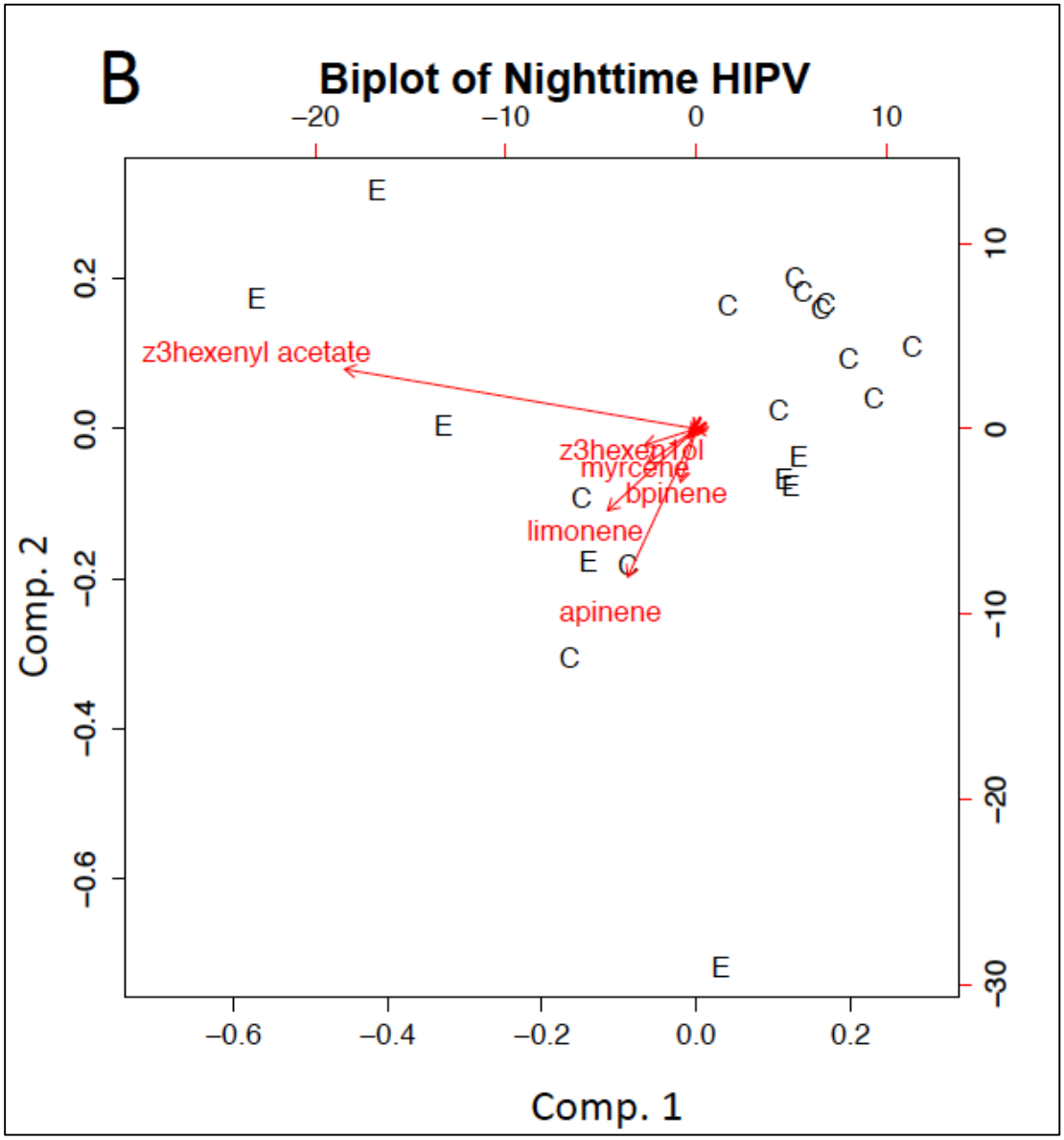

Figure 3-4
Biplot of first two principle components of the scotophase herbivore-induced volatiles for *E. solidaginis* emission-exposed and unexposed *S. altissima.* Arrows indicate the weight given to individual compounds. Not all compound labels are shown for legibility. Individual plants are labeled with a character representing the treatment (C = control, E = exposed).

	Daytime HIPV		Nighttime HIPV	
	Exposure treatment			
	Eurosta	Control	*Eurosta*	Control
Z. mays Volatile Compounds	Induced VOC (ng cm^{-2}) ± SE	Induced VOC (ng cm^{-2}) ± SE	Induced VOC (ng cm^{-2}) ± SE	Induced VOC (ng cm^{-2}) ± SE
(*Z*)-3-hexen1ol	0.77 ± 0.55	1.03 ± 0.73	0.00 ± 0.00	0.00 ± 0.00
Unknown 1	8.17 ± 4.01	6.34 ± 5.96	0.00 ± 0.00	0.00 ± 0.00
Unknown 2	4.54 ± 3.79	3.07 ± 5.10	0.00 ± 0.00	0.00 ± 0.00
Unknown 3	8.66 ± 5.88	16.08 ± 15.5	0.00 ± 0.00	0.00 ± 0.00
Myrcene	-3.59 ± 2.17	-1.59 ± 4.24	0.00 ± 0.00	0.00 ± 0.00
(*Z*)-3-Hexenyl acetate	3.48 ± 1.24	5.20 ± 2.38	3.71 ± 2.05	3.79 ± 3.72
Limonene	-0.08 ± 0.10	0.23 ± 0.15	-0.45 ± 0.48	-0.48 ± 0.74
(*E*)-β-ocimene	-0.02 ± 0.15	-0.02 ± 0.16	0.36 ± 0.33	0.25 ± 0.61
Linalool	1.04 ± 0.52	2.73 ± 1.20	20.96 ± 13.2	41.05 ± 34.5
Nonatriene	0.78 ± 0.38	1.45 ± 0.55	9.53 ± 5.26	12.73 ± 10.9
(*Z*)-3-hexenyl isobutyrate	-0.15 ± 0.22	-0.10 ± 0.23	-1.04 ± 1.81	-2.43 ± 4.07
(*Z*)-3-hexenyl butyrate	-0.22 ± 0.15	0.00 ± 0.00	-0.18 ± 0.30	0.02 ± 1.01
(*E*)-2-hexenyl butyrate	-0.22 ± 0.15	0.00 ± 0.00	0.40 ± 0.17	0.70 ± 0.53
Bornyl acetate	-0.28 ± 0.29	0.00 ± 0.00	-0.17 ± 0.18	0.02 ± 0.44
(*Z*)-jasmone	-0.08 ± 0.20	-0.05 ± 0.14	-0.97 ± 0.71	-0.56 ± 1.06
Caryophyllene	0.96 ± 0.65	1.76 ± 0.72	12.82 ± 9.31	12.33 ± 10.5
α-humulene	1.77 ± 0.85	3.62 ± 1.29	20.83 ± 14.78	25.03 ± 25.7
β-farnescene	0.03 ± 0.03	0.05 ± 0.05	-1.52 ± 1.40	-1.44 ± 2.71
Unknown 4	7.41 ± 6.99	9.39 ± 10.84	0.00 ± 0.00	0.00 ± 0.00
α-farnescene	-2.43 ± 1.29	-2.54 ± 3.92	0.00 ± 0.00	0.00 ± 0.00
Tridecatetraene	0.15 ± 0.2	0.29 ± 0.16	-1.15 ± 2.39	-0.70 ± 4.57
Indole	5.0 ± 4.07	4.98 ± 2.44	15.35 ± 10.55	18.26 ± 17.8

Table S2

Day and night *Zea mays* individual herbivore-induced volatile organic compounds. Table showing the individual compounds that make up the volatile blend of herbivore-damaged *Z. mays* plants. (VOC; means ± standard error; untransformed data shown). Herbivore-induced volatiles were calculated by subtracting the undamaged volatile production from the herbivore-damaged volatile production (damaged VOC- undamaged VOC). Negative values indicate these compounds were emitted in lower amounts following herbivore-feeding damage.

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Chapter 4

A single compound in the goldenrod gall fly (*Eurosta solidaginis*) volatile emission primes anti-herbivore defenses in tall goldenrod (*Solidago altissima*)

Abstract

Olfactory cues play important roles in many ecological interactions, especially among plants and insects. Some of the best-documented examples include pheromonal communication among conspecific insects and floral scents attracting insect pollinators. Additionally, studies have revealed that plants also perceive olfactory cues. For example, parasitic plants in the genus *Cuscuta* grow toward the odor of their host plants and several studies have demonstrated that plants can eavesdrop on olfactory cues emitted by their insect-damaged neighbor plants, responding by priming their own anti-herbivore defenses. I recently discovered for the first time that some plants can also perceive and respond to olfactory cues emitted directly by insect herbivores. My findings revealed that tall goldenrod (*Solidago altissima*) plants exhibit enhanced defense responses following exposure to the putative sex attractant of a specialist herbivore, the goldenrod gall fly (*Eurosta solidaginis*). Here I build on my previous work and show that the most abundant compound in the *E. solidaginis* emission ((5*S*,7*S*)-7-methyl-1,6-dioxasopiro[4.5]decane) is sufficient and necessary to elicit a priming response in *S. altissima* plants exposed to the emission. I also found that the second compound, 1-nonanol, does not prime *S. altissima* defenses. A racemic mix of two other spiroacetals in the blend seems to have

triggered an intermediate response that is statistically not distinguishable from the *E. solidaginis* blend or control.

Introduction

All plants risk attack by insects and other herbivores. As a consequence, plants have evolved a variety of defensive strategies to combat herbivore attack, including production of defensive chemicals (131, 132). Because such defenses may be costly to produce and maintain, plants may only induce anti-herbivore defenses when they are attacked rather than expressing them constitutively (65, 100, 131, 133–135). It has been well established that insect feeding damage triggers plant defenses; however, more recent discoveries have revealed that plants also respond to environmental cues associated with the presence of insect herbivores, allowing them to express or prime their defenses for future attack. These herbivore-associated cues may be physical or biochemical indicators of the presence of herbivores or their eggs. For example, insect footsteps, broken trichomes, or oviposition have been found to initiate plant defenses (136–139). Additionally, some plants perceive olfactory cues associated with nearby herbivore-damaged plants and respond by priming their own anti-herbivore defenses. Undamaged plants exposed to airborne chemical cues emitted by insect-damaged neighbors, or damaged parts of the same plant, exhibit an enhanced defense response when challenged by insect herbivores (101, 102, 104, 107, 140, 141).

Broadening the range of cues that plants can exploit, my recent work demonstrated that plants can also perceive odors emitted directly by insect herbivores and

respond by priming their anti-herbivore defenses (83, 84). I found that tall goldenrod (*Solidago altissima*) plants exposed to the putative sex attractant of the specialist goldenrod gall fly (*Eurosta solidaginis*) received less herbivore damage in both field and laboratory studies than plants not exposed to the fly emission. This included damage from *E. solidaginis* flies as well as from a suite of different herbivores. Our results also indicated that *S. altissima* plants previously exposed to the *E. solidaginis* emission induced substantially higher amounts of jasmonic acid, a key defense phytohormone, and emitted greater quantities of defense-related volatile compounds following herbivore damage (83, 84). Taken together, these results provide strong evidence that *S. altissima* plants perceived the *E. solidaginis* emission and responded by enhancing their anti-herbivore defenses, thus documenting a new class of chemically mediated interactions between plants and insects.

Because little is known about the mechanisms underlying plant perception of olfactory cues or the specificity of plant olfaction, the aim of the current study was to determine which individual compounds in the blend of volatiles emitted by *E. solidaginis* elicit a response from *S. altissima* plants. Many previous studies have documented plant responses to olfactory cues, but only a handful have identified specific compounds that trigger plant responses to odors. A majority of these studies have focused on plant perception of green leaf volatile compounds, which are 6-carbon alcohols, aldehydes, and esters induced by herbivore feeding damage (67). Maize (*Zea mays*), hybrid poplar (*Populus deltoides x nigra*), tomato (*Lycopersicon esculentum*), and lima bean (*Phaseolus lunatus*) plants primed or induced anti-herbivore defenses following exposure to green leaf volatiles, including (*Z*)-3-hexenyl acetate, (*Z*)-3-hexanal, and (*Z*)-3-hexanol

(104, 141–143). More recently, the aromatic herbivore-induced volatile compound, indole, was identified as an important priming signal in maize plants (144). Additionally, parasitic plants in the genus *Cuscuta* grow toward the terpenes β-phellandrene, β-myrcene, and α-pinene, which are part of the odor blend emitted by their host plant, tomato (145).

Male *E. solidaginis* flies emit very large quantities ($\geq 70 \pm 20$ µg over 24 h) of a volatile emission that is attractive to female *E. solidaginis* (83). The putative *E. solidaginis* sex attractant comprises two major compounds, the spiroacetal (5*S*,7*S*)-7-methyl-1,6-dioxasopiro[4.5]decane, and the long-chain alcohol, 1-nonanol. The emission also contains several other spiroacetals including (*E*) and (*Z*)-2-methyl-1,6-dioxaspiro[4.5]decane. (5*S*,7*S*)-7-methyl-1,6-dioxasopiro[4.5]decane is the most abundant compound in the blend, followed by 1-nonanol. These two compounds, together with a third spiroacetal that is likely (*E*)-2-methyl-1,6-dioxaspiro[4.5]decane make up more than 95% of the *E. solidaginis* emission.

To determine which compounds in the *E. solidaginis* emission elicit a priming response in *S. altissima*, I exposed plants to the individual compounds composing the blend and examined their influences on *S. altissima* defenses. Similar to my initial experiments with the crude extract of the *E. solidaginis* emission (83, 84), I conducted insect feeding assays using goldenrod leaf beetles (*Trirhabda virgata*) on plants exposed to the total blend, a synthetic version of individual compounds in the blend, or a solvent control. I also measured levels of jasmonic acid in these plants before and after insect feeding damage to determine whether individual compounds elicited a similar

biochemical defense response in *S. altissima* to those exposed to the entire emission blend.

Results

Conopthorin Experiment

To determine whether the most abundant compound in the *E. solidaginis* emission ((5*S*,7*S*)-7-methyl-1,6-dioxasopiro[4.5]decane) is sufficient and necessary to elicit an enhanced defense response in *S. altissima* plants, I exposed *S. altissima* plants to either the total *E. solidaginis* emission, commercially available conopthorin (a racemic mix of (5*S*,7*S*)- and (5*S*,7*R*)-7-methyl-1,6-dioxasopiro[4.5]decane), or a dichloromethane solvent control and examined the influence on plant defensive responses. In a greenhouse insect-feeding experiment, I found that *S. altissima* plants exposed to either the total *E. solidaginis* emission blend or conopthorin received significantly less feeding damage from *T. virgata* beetles compared to control plants (Fig. 1; ANOVA $F_{1,21} = 18.5$, $P = 0.0002$). I also found that plants exposed to either the total *E. solidaginis* blend or conopthorin exhibited an enhanced defense response, as they induced significantly higher levels of the defense phytohormone jasmonic acid following insect damage (Fig. 2; repeated-measures ANOVA time factor, $F_{2,21} = 21.3$, $P < 0.00001$; treatment x time interaction, $F_{2,21} = 4.7$, $P = 0.0002$).

Individual Compound Experiment with Additional Compounds

To determine whether pure (5*S*,7*S*)-7-methyl-1,6-dioxasopiro[4.5]decane or other individual compounds in the *E. solidaginis* emission prime *S. altissima* defenses, I conducted a similar experiment to the one described previously, this time exposing *S. altissima* plants to either the total *E. solidaginis* emission, pure (5*S*,7*S*)-7-methyl-1,6-dioxasopiro[4.5]decane, 1-nonanol, a racemic mix of (*E*) and (*Z*)-2-methyl-1,6-dioxaspiro[4.5]decane or a dichloromethane solvent control. As in the previous experiment, I found that *T. virgata* beetles consumed significantly less leaf tissue on plants exposed to the total *E. solidaginis* emission or pure (5*S*,7*S*)-7-methyl-1,6-dioxasopiro[4.5]decane compared to control plants. Plants exposed to 1-nonanol experienced similar levels of damage compared to controls, while plants exposed to the mix of (*E*) and (*Z*)-2-methyl-1,6-dioxaspiro[4.5]decane received an intermediate level of damage that was not statistically distinguishable from either the *E. solidaginis* emission-exposed or control plants (Fig. 3; Table 1; ANOVA $F_{4,25} = 8.84$, $P = 0.0015$). I also found that plants exposed to either the total *E. solidaginis* blend or pure (5*S*,7*S*)-7-methyl-1,6-dioxasopiro[4.5]decane exhibited an enhanced defense response, as they induced significantly higher levels of jasmonic acid (JA) following insect damage (Fig. 4; Table 2; ANOVA $F_{4,25} = 12.8$, P=0.0003). 1-nonanol-exposed *S. altissima* induced levels of JA that were not significantly different from control plants and were significantly lower than for *E. solidaginis* emission- or (5*S*,7*S*)-7-methyl-1,6-dioxasopiro[4.5]decane-exposed plants. The quantity of jasmonic acid induced in plants exposed to (*E*) and (*Z*)-2-methyl-1,6-dioxaspiro[4.5]decane was not significantly different from any other treatment.

Discussion

Our results indicate that the most abundant compound in the *E. solidaginis* emission, (5*S*,7*S*)-7-methyl-1,6-dioxasopiro[4.5]decane, is sufficient and necessary to prime anti-herbivore defenses in *S. altissima* plants exposed to the *E. solidaginis* emission. This study presents evidence from herbivore damage and inducible chemical defenses demonstrating that *S. altissima* exposed to the emission or the most abundant compound in the emission exhibit an enhanced defense response and subsequently receive less herbivore-feeding damage. *T. virgata* beetles consumed approximately half as much leaf tissue on plants exposed to conopthorin or pure (5*S*,7*S*)-7-methyl-1,6-dioxasopiro[4.5]decane compared to control plants, which was a similar reduction in feeding observed for plants exposed to the entire *E. solidaginis* emission (Fig. 1, 3, Table 1). In the first experiment, I also found that *S. altissima* plants exposed to conopthorin or the *E.* solidaginis emission induced several times more jasmonic acid compared to unexposed control plants after feeding damage by *T. virgata*, indicating an enhanced inducible defense response (Fig. 2). This was also true in the second experiment, where plants exposed to pure (5*S*,7*S*)-7-methyl-1,6-dioxasopiro[4.5]decane or the *E. solidaginis* emission induced significantly more jasmonic acid compared to control plants, although overall JA levels were lower (Fig. 4, Table 2). In the second experiment, a different genotype of *S. altissima* was used, which may explain the overall lower induction of JA. Because the enhanced induction of jasmonic acid was observed in exposed plants following herbivore damage, but not in undamaged *S. altissima*, we can infer that

exposure to the *E. solidaginis* emission (or the most abundant compound in the emission) primes, but does not directly induce, *S. altissima* defenses.

The second most abundant compound in the *E. solidaginis* emission, the long-chain alcohol, 1-nonanol, does not appear to play a role in priming *S. altissima* defenses. *T. virgata* beetles consumed similar amounts of leaf tissue on plants exposed to 1-nonanol compared to control plants, and did not induce levels of JA that were significantly different from control plants (Fig.3, 4, Table 1, 2). These findings indicate that exposure to 1-nonanol did not alter the plants' palatability or defense responses. This finding is of interest because although 1-nonanol is emitted in relatively high abundances by *E. solidaginis*, it does not appear to be a perceivable olfactory cue for *S. altissima* plants. This provides some insight into the specificity of *S. altissima* olfactory perception and suggests the presence of a spiroacetal-specific receptor.

Two other spiroacetals in the *E. solidaginis* emission, (*E*) and (*Z*)-2-methyl-1,6-dioxaspiro[4.5]decane, appear to elicit an intermediate defense response that is statistically indistinguishable from control or emission exposed plants (Fig. 3, 4, Table 1, 2). I observed a trend toward *T. virgata* beetles consuming less leaf tissue on these plants than control plants, but more than on emission-exposed plants (Table 1). The level of JA induced in these plants was not statistically different than the other treatments (Table 2). One possible explanation is that a racemic mix of (*E*) and (*Z*)-2-methyl-1,6-dioxaspiro[4.5]decane was used for this treatment. Depending on the specificity of *S. altissima* perception of spiroacetal olfactory cues, a racemic mix of compounds could have diluted the effect by reducing the number of perceivable molecules. An additional experiment focusing on specific, individual spiroacetal compounds is needed to further

elucidate this specificity and to determine if *S. altissima* truly perceive spiroacetals other than (5*S*,7*S*)-7-methyl-1,6-dioxasopiro[4.5]decane.

These findings are noteworthy because little is known about how plants perceive olfactory cues or the specificity of plant perception of odor cues. Only a few studies to date have attempted to track plant perception of specific volatile compounds and these studies have focused exclusively on plant-produced odors (104, 141–145). The current study documents the first known example of plants perceiving insect-derived odors and demonstrates that a single compound is sufficient to elicit a response in goldenrod plants. Many of the previous studies investigating olfactory cues involved in plant olfaction and priming of anti-herbivore defenses have concentrated on the role of green leaf volatiles (GLVs) (104, 141–143). Several GLVs have been found to prime or induce plant defense responses, however, because these compounds are emitted in response to any physical tissue damage, they may be less specific and less reliable cues for plants to predict future herbivore attack. In contrast, herbivore-specific cues such as those emitted only in response to actual herbivore feeding, or in the case of this study, are emitted directly by insect herbivores, are likely to provide more specific and reliable warnings of future damage (144).

The prevailing theory behind the evolution of plant perception of olfactory cues and plant defense priming proposes that plant olfaction originated as a form of within plant signaling to overcome vascular constraints, with eavesdropping on neighbor plants as a secondary consequence (64, 101–103). The current study is the first example of plants perceiving olfactory cues not emitted by other plants or parts of the same plant and thus adds a new level of complexity to the subject. It is possible that *S. altissima*

perception of olfactory cues also evolved as a form of within-plant signaling and has since expended to include the odor of a specialist herbivore. It is also possible that *S. altissima* perception of the *E. solidaginis* emission evolved independently from its ability to perceive plant-produced odors. Additional studies are needed to elucidate the mechanisms of olfaction in *S. altissima* and determine their evolutionary origins.

Tall goldenrod (*S. altissima*) has the potential to provide a suitable system for studying plant olfaction and elucidating mechanisms and receptors. Based on the findings from the current study, it appears that *S. altissima* plants respond strongly to a single spiroacetal compound, indicating that the response could be fairly specific. Some progress has been made on identifying specific olfactory cues involved in plant perception (104, 141–144). However, very few studies to date have also identified a mechanism accompanying the perception and processing of olfactory signals, one being the volatile plant hormone ethylene and the other being the GLV, (*Z*)-3-hexenol (143, 146, 147). It is unlikely that the same mechanisms apply to all plant species and all olfactory cues. Other studies have suggested the involvement of receptor-like kinases, however future work is needed to confirm their possible role (148).

Methods and Materials

The Study System

The goldenrod gall fly (*E. solidaginis*) induces sphere-shaped galls on stems of its host plant, tall goldenrod (*S. altissima*). After overwintering as larvae inside of galls, *E. solidaginis* flies typically pupate and emerge as adults in May in the Northeastern United

States. Male flies perch on the apices goldenrod plants and attempt to attract females by rocking their bodies side to side, spreading their wings, and emitting large quantities of a putative sex pheromone (83, 84, 149, 150). After mating, female flies seek suitable oviposition sites in the apical buds of tall goldenrod plants. *E. solidaginis* eggs typically hatch within a week and larval feeding induces the formation of galls that become visible within three weeks (149, 150). *E. solidaginis* galls reduce the reproductive fitness of *S. altissima* plants and divert resources away from leaf and rhizome growth as well as inflorescence and achene production (150, 151).

Plants and Insects

I grew tall goldenrod (*S. altissima*) plants from rhizomes of the RV2 and REI clone lines in insect-free, climate-controlled greenhouses, with supplemental lighting (metal halide and high pressure sodium lights). (16 h light: 8 h dark; 24°C: 21°C; 60%RH). Rhizomes for these experiments were collected near State College, PA, USA and were washed and stored at 4°C prior to planting. I cut rhizomes of similar diameter into 5 cm segments and planted them in trays with peat-based potting soil (Pro-Mix BX; Premier Horticulture Inc., Quakertown, PA, USA). Approximately two weeks later, I transplanted sprouted ramets into individual pots (16 cm diameter, 16.5 cm tall) using the same potting soil and added 0.5tsp Osmocote fertilizer (8– 45– 14 N– P– K, Scotts, Marysville, OH, USA) to each pot. *S. altissima* plants used in experiments were 7 wk old and ~ 25 cm tall.

I obtained adult male *E. solidaginis* flies from overwintering galls collected near State College, PA, USA. I placed the galls at in a climate-controlled incubator (16 h light:

8 h dark; 22°C, 20°C; 65% RH) for approximately 3 wk to induce pupation and adult emergence.

Adult goldenrod leaf beetles (*Trirhabda virgata*) were collected from fields containing goldenrod near State College, PA, USA. Larvae for experiments were obtained by collecting eggs from adult beetles, storing them at 4°C for three to five months and then placing the eggs in a climate-controlled greenhouse (16 h light: 8 h dark; 24°C: 21°C; 60%RH) (152). Larvae and adult beetles were fed greenhouse-grown goldenrod plants until they were used in experiments.

Collection of the *E. solidaginis* Emission

I collected the male *E. solidaginis* emission by aerating newly emerged adult male flies in small glass chambers for 24 h (83, 84). Using a push-pull volatile collection system, I pushed clean air into the chambers at 0.6 L min^{-1} and pulled air out of the chambers, over two adsorbent filters containing 45 mg of Super-Q (Alltech Associates, Deerfield, IL, USA) at 0.5 L min^{-1}. I eluted the filters using 150 µL dichloromethane. Individual samples were pooled to ensure a uniform concentration of emission for the exposure treatments. A 150 µL aliquot of the emission solution was taken for analysis and 5 µ L of a standard containing nonyl acetate (80 ng/µL) and n-octane (40 ng/µL) was added. I quantified amounts of each compound in the samples using an Agilent model 7890A gas chromatograph fitted with a flame ionization detector, using a splitless injector held at 220°C. The column (HP-5, 15 mÅ~ 0.25 mmÅ~ 0.25 µ m film thickness; J&W Scientific, Folsom, CA) was maintained at 35 °C for 30 s, then ramped 2°C min^{-1} to 130°C, and ramped again at 20°C min−1 to 220°C.

Individual Compounds in the *E. solidaginis* Emission

The compounds that compose the *E. solidaginis* emission were identified with help from Hans Alborn (US Department of Agriculture, Agricultural Research Service, Chemistry Research Unit, Gainesville, FL, USA) and Wittko Francke (University of Hamburg, Institute for Organic Chemistry, Hamburg, Germany) using gas chromatography coupled with a mass spectrometer. Male *E. solidaginis* flies were found to emit ~70± 20 μg based on 24-h headspace aerations of eight males (83). The blend of this putative sex attractant is dominated by the spiroacetal (5*S*,7*S*)-7-methyl-1,6-dioxasopiro[4.5]decane and the long-chain alcohol, 1-nonanol. The emission also contains several other spiroacetals, including (*E*)-2-methyl-1,6-dioxaspiro[4.5]decane, and (*Z*)-2-methyl-1,6-dioxaspiro[4.5]decane. Together, these compounds account for ~95% of the total emission.

Wittko Francke (University of Hamburg, Institute for Organic Chemistry, Hamburg, Germany) synthesized pure (5*S*,7*S*)-7-methyl-1,6-dioxasopiro[4.5]decane and a racemic mix of (*E*)-2-methyl-1,6-dioxaspiro[4.5]decane, and (*Z*)-2-methyl-1,6-dioxaspiro[4.5]decane. 1-nonanol (purity $\geq$ 98.0%) was purchased from Sigma-Aldrich (St. Louis, MO, USA). Conopthorin, a racemic mix of (5*S*,7*S*)-7-methyl-1,6-dioxasopiro[4.5]decane and (5*S*,7*R*)-7-methyl-1,6-dioxasopiro[4.5]decane, was purchased from Contech Enterprises Inc. (Victoria, BC, Canada). The *E. solidaginis* emission crude extract solution was ~0.8 μg μL^{-1} and diluted in dichloromethane. The conopthorin, *(5S,7S)*-7-methyl-1,6-dioxasopiro[4.5]decane, 1-nonanol, and (*E*)-2-methyl-1,6-dioxaspiro[4.5]decane, and (*Z*)-2-methyl-1,6-dioxaspiro[4.5]decane solutions were ~0.6 μg μL^{-1} and diluted in dichloromethane.

Emission Exposure Treatments

I exposed *S. altissima* plants to the various treatments by placing individual plants in 4-L volume glass chambers that rested on Teflon bases. The glass domes and Teflon bases were cleaned with soap and water and then rinsed with acetone and finally hexanes. The stems of the plants were wrapped in clean cotton where they passed through a hole in the center of the Teflon bases. Clean, filtered air was pushed into each chamber at a rate of 2.0 L min^{-1} for the first experiment and 2.5 L min^{-1} for the second experiment. This airflow was added to prevent accumulation of condensation and an unrealistic concentration of the *E. solidaginis* compounds from building up inside the chambers. I allowed the plants to acclimate to the chambers for approximately 2 hours before beginning the exposure treatments. For the first experiment, I added 45 μL of *E. solidaginis* emission crude extract, conopthorin, or dichloromethane to rubber septa. For the second experiment, I added 45 μL of *E. solidaginis* emission crude extract, (5*S*,7*S*)-7-methyl-1,6-dioxasopiro[4.5]decane, 1-nonanol, a mix of mix of (*E*)-2-methyl-1,6-dioxaspiro[4.5]decane, and (*Z*)-2-methyl-1,6-dioxaspiro[4.5]decane, or dichloromethane to rubber septa. These values represent six-hour equivalents emitted by male flies. I added two rubber septa every six hours for twenty-four hours to space out the exposure.

Feeding Assays

I conducted insect herbivore feeding assays as previously described in Helms et al 2013 and Helms et al 2014 to determine the influence of exposure to the individual *E. solidaginis* compounds on *S. altissima* anti-herbivore defenses (83, 84). After exposing

plants to the various treatments for twenty-four hours, I allowed *Trirhabda virgata* beetles to feed on the plants for an additional twenty-four hours. For the first experiment, adult beetles were starved for twenty-four hours at room temperature before the experiment. For the second experiment, *T. virgata* larvae were starved for four hours at room temperature before the experiment. After adult beetles or larvae fed for twenty-four hours, I harvested the plants and scanned the leaves to quantify the total leaf area consumed. I quantified the total area removed using Adobe Photoshop.

Quantification of Jasmonic Acid

I measured levels of Jasmonic acid (JA) as an indicator of induced plant defenses. After plants were exposed to the various treatments for twenty-four hours, I harvested one undamaged leaf from each plant (~100 mg tissue). Leaves from the upper-middle section of the plant, of similar size were harvested. Then after beetles fed on the plants for twenty-four additional hours, I harvested a recently damaged leaf from each plant, again from the upper-middle of the plant, preferably with similar damage levels where beetles were observed actively feeding. The tissue was flash frozen in liquid nitrogen and stored at -80°C until analyzed. To quantify jasmonic acid, I used the procedure described by Schmelz et al 2003, Schmelz et al 2004 (153, 154). Briefly, endogenous plant hormones were derivatized to methyl esters and were isolated using vapor-phase extraction. These compounds were then analyzed by coupled GC-MS with isobutene chemical ionization using selection-ion monitoring. I quantified amounts of jasmonic acid relative to an internal standard dihydro-JA, 100 ng of which was added to each

sample. I confirmed the identity of methyl jasmonate, I compared the retention times and spectra of our samples with standards of the pure compound.

Statistical Analyses

Statistical analyses were performed using the software program R (R Development Core Team (2015)). I analyzed data from the insect-feeding assays using a one-way ANOVA, after confirming that the data met the assumptions of normality and equal variance. Data from the second feeding experiment were log-transformed, and JA-data were square-root transformed to meet these assumptions. Tukey's Honest Significant Differences Test was used to conduct the post-hoc multiple comparisons analysis. The conopthorin experiment JA data were log-transformed and analyzed using repeated-measures ANOVA with the appropriate post-hoc multiple comparisons using contrasts and generalized least squares (36).

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Figures and Tables

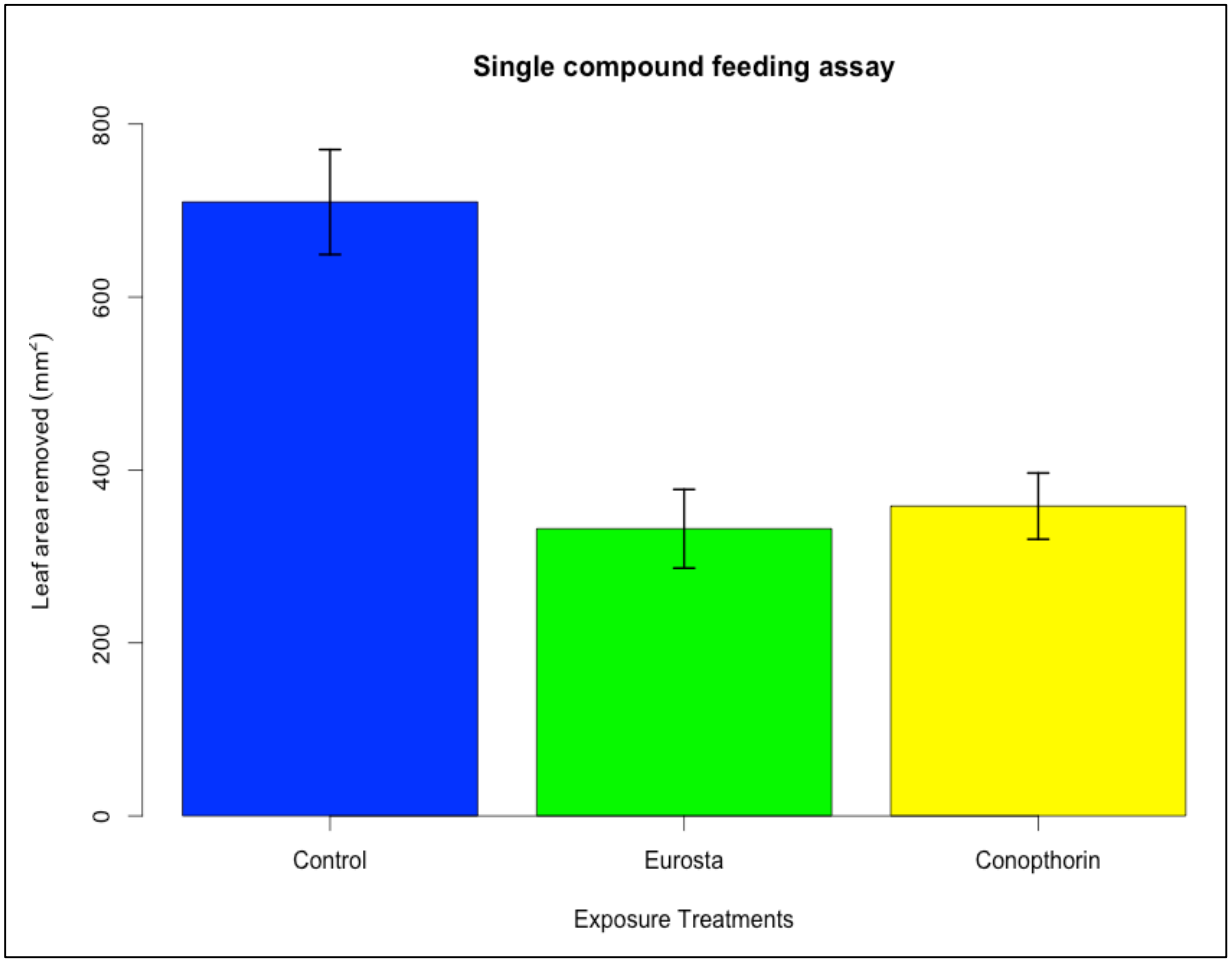

Figure 4-1
Feeding damage by *T. virgata* on *S. altissima*. Adult *T. virgata* beetles consumed significantly less leaf tissue on plants exposed to the *E. solidaginis* emission blend or conopthorin compared to solvent controls (ANOVA $F_{1,21} = 18.5$, $P = 0.0002$).

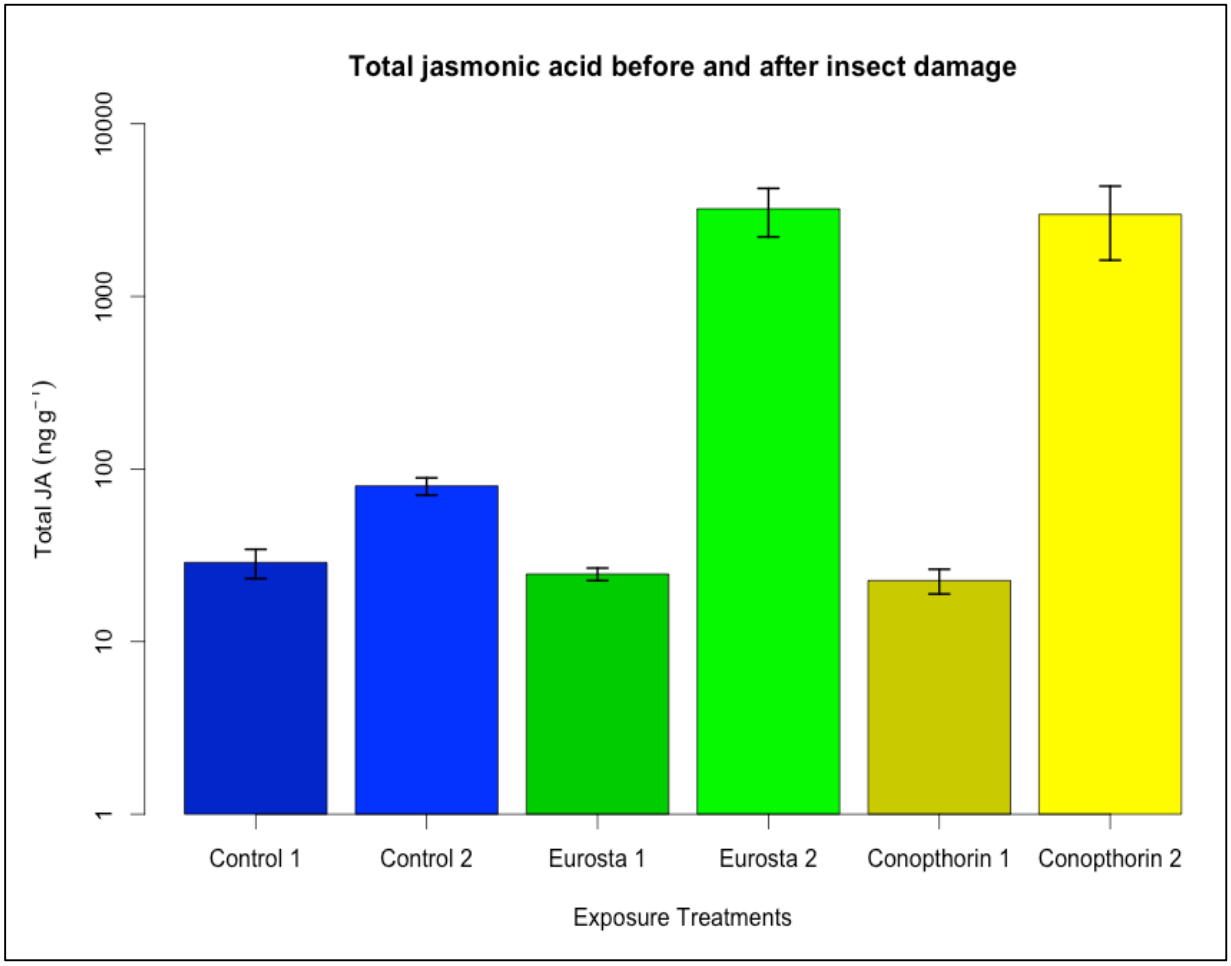

Figure 4-2
Levels of JA in *S. altissima* leaves following exposure to the emission of male *E. solidaginis*, conopthorin, or a solvent control, before and after herbivory by *T. virgata* beetles. After twenty-four hours of feeding damage, induced-JA levels were significantly higher in emission-exposed and conopthorin-exposed plants (repeated-measures ANOVA time factor, $F_{2,21} = 21.3$, $P < 0.00001$; treatment x time interaction, $F_{2,21} = 4.7$, $P = 0.0002$). The darker bars represent undamaged leaves from each treatment and the lighter bars represent damaged leaves from each treatment. The data shown are untransformed, but analyses were conducted on log-transformed data and the results are shown with a log scale on the y-axis.

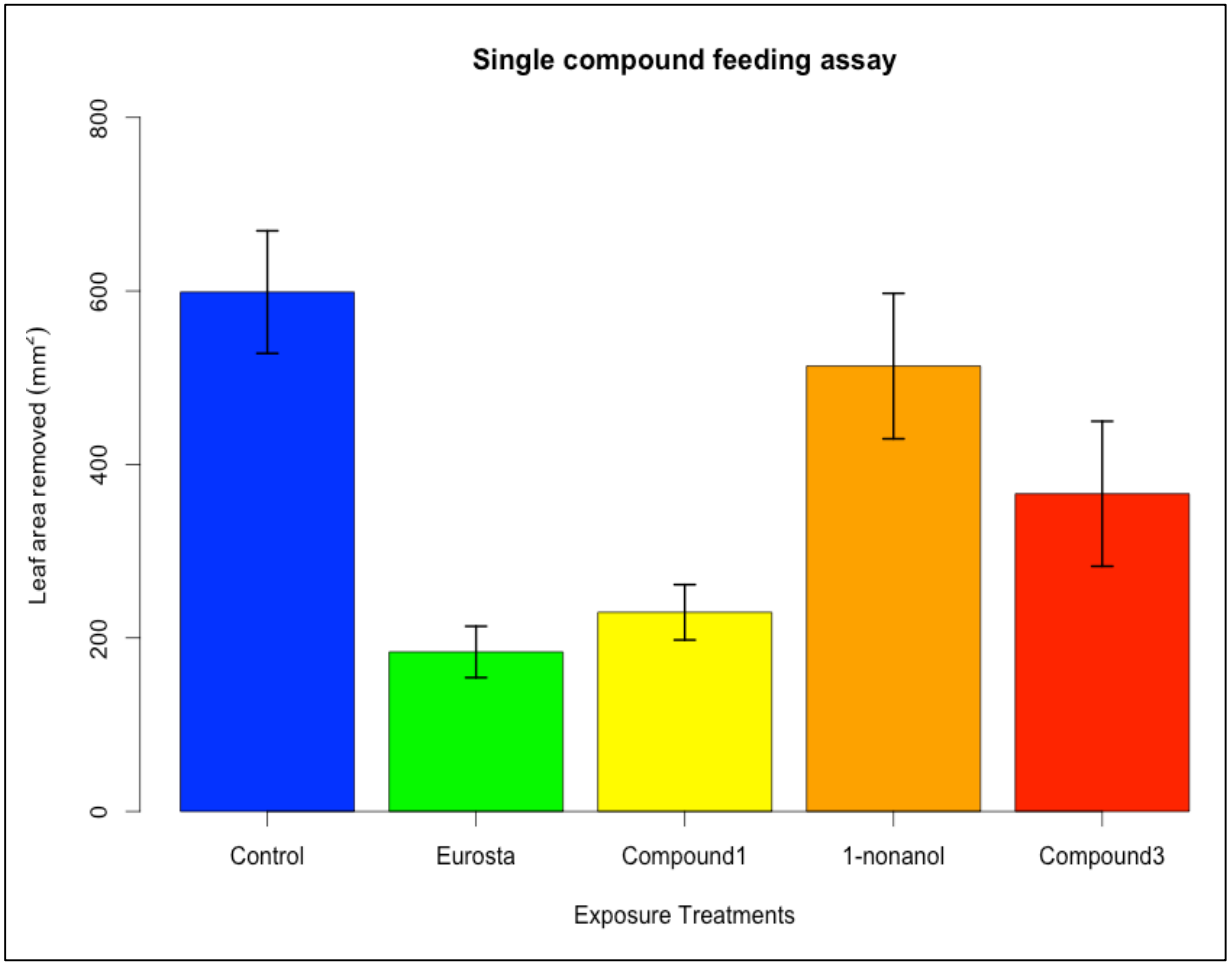

Figure 4-3
Feeding damage by *T. virgata* on *S. altissima*. *T. virgata* larvae consumed significantly less leaf tissue on plants exposed to the *E. solidaginis* emission blend or the most abundant compound in the blend, (5*S*,7*S*)-7-methyl-1,6-dioxasopiro[4.5]decane, compared to solvent controls. *S. altissima* exposed to the second most abundant compound, 1-nonanol, received a similar amount of damage to control plants and plants exposed to a mix of (*E*) and (*Z*)-2-methyl-1,6-dioxaspiro[4.5]decane) had an intermediate reduction in feeding damage (ANOVA $F_{4,25} = 8.84$, $P = 0.0015$). Data shown are not transformed but statistical analyses were performed on log-transformed data.

Treatment Comparison	**Difference in Means**	***P*-value**
Control-*Eurosta*	414.83	0.001*
Control-Compound1	369.12	0.004*
Control-1-nonanol	85.31	0.88
Control-Compound3	232.81	0.11
Eurosta-Compound1	-45.71	0.99
Eurosta-1-nonanol	-329.52	0.01*
Eurosta-Compound3	-182.32	0.30
Compound1-1-nonanol	-283.81	0.04*
Compound1-Compound3	-136.61	0.58
1-nonanol-Compound3	147.2	0.51

Table 1

This table shows multiple comparisons from the feeding assay with multiple individual compounds. The "Difference in Means" values are for non-transformed data. Statistical analyses were performed on log-transformed data to meet the assumptions of normality and equal variance. *P-value* with an asterisk indicates significant difference ($P \leq 0.05$).

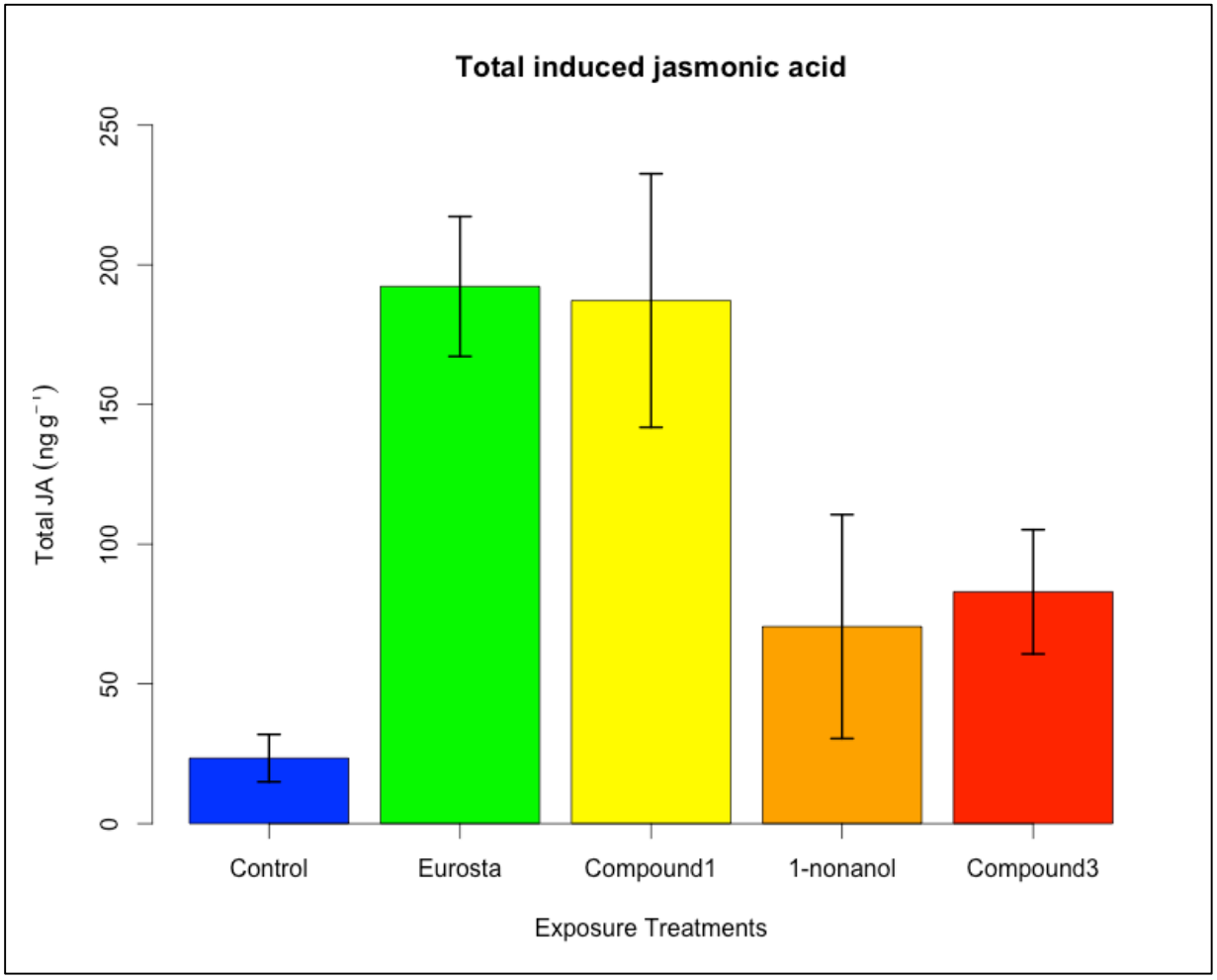

Figure 4-4
Jasmonic acid induced by *T. virgata* on *S. altissima*. *T. virgata* feeding damage induced significantly more JA in plants exposed to the *E. solidaginis* emission blend or the most abundant compound in the blend, (5*S*,7*S*)-7-methyl-1,6-dioxasopiro[4.5]decane, compared to solvent controls. *S. altissima* exposed to the second most abundant compound, 1-nonanol, induced a similar amount of JA to control plants and plants exposed to a mix of (*E*) and (*Z*)-2-methyl-1,6-dioxaspiro[4.5]decane) had an intermediate JA induction (ANOVA $F_{4,25} = 12.8$, P=0.0003). Data shown are not transformed but statistical analyses were performed on square root-transformed data.

Treatment Comparison	**Difference in Means**	***P*-value**
Control-*Eurosta*	-168.8	0.004*
Control-Compound1	-163.8	0.008*
Control-1-nonanol	-47.1	0.92
Control-Compound3	-59.5	0.44
Eurosta-Compound1	5.0	0.99
Eurosta-1-nonanol	121.7	0.03*
Eurosta-Compound3	109.3	0.19
Compound1-1-nonanol	116.7	0.05*
Compound1-Compound3	104.3	0.28
1-nonanol-Compound3	-12.4	0.90

Table 2

This table shows multiple comparisons from the jasmonic acid induction experiment with individual compound exposure. The "Difference in Means" values are for non-transformed data. Statistical analyses were performed on square root-transformed data to meet the assumptions of normality and equal variance. *P-value* with an asterisk indicates significant difference ($P \leq 0.05$).

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Chapter 5

Characterization of male *Eurosta solidaginis* volatile emission release and evidence of sexual selection

Abstract

Many insect species use pheromones to attract and locate mates. I recently discovered that male goldenrod gall flies (*Eurosta solidaginis*) emit large quantities of a volatile emission that appears to function as a sex pheromone and is attractive to female flies. This emission comprises two main compounds, (7*S*,5*S*)-7methyl-1,6-dioxaspiro[4.5]decane, 1-nonanol, as well as several additional spiroacetals that include (*E*) and (*Z*) 2-methyl-1,6-dioxaspiro[4.5]decane. Recent research from my dissertation also revealed that tall goldenrod (*Solidago altissima*) plants eavesdrop on the *E. solidaginis* emission and respond by priming their anti-herbivore defenses. Because eavesdropping by goldenrod plants has a negative fitness consequence for *E. solidaginis* flies, one would expect natural selection to favor low-emitting males. However, if mate attraction and mating success depend on emission of this putative pheromone, one might expect sexual selection leading to high-emitting males. In this study, I examined traits typically associated with male quality and the emission release for each male to determine whether emission production is an honest signal of male quality and might be the result of sexual selection. I found that production of the male fly emission is not correlated with any of the traits I measured. Interestingly, I also observed a significant change in the ratio of (7*S*,5*S*)-7methyl-1,6-dioxaspiro[4.5]decane to 1-nonanol in older virgin males compared to newly emerged virgin males.

Introduction

The topic of sexual selection has fascinated ecologists and evolutionary biologists for more than one hundred fifty years since Charles Darwin first proposed the theory. According to Darwin, sexual selection is not a struggle for existence, but instead a struggle among males for access to females, and the result is not death, but leaving few or no offspring (125). Generally, the most vigorous males in a population, or those best adapted to their environment, will leave the most progeny. In some cases, however, general vigor for males may be less important than having traits that confer an advantage in the population when competing for females, such as ornamentation that attracts females or special weaponry that helps dominate other males. Preference for these traits by females is sexual selection, which can drive development of traits, occasionally producing extreme phenotypes that can be costly to produce and maintain, but do not contribute directly to an organism's survival. These traits persist because they help organisms attract and secure mates, thereby increasing that organism's reproductive fitness (125, 155, 156).

Over the past century, several theories have emerged to explain different cases of sexual selection. One theory, Fisherian runaway selection, predicts that female preference can overcome natural selection against a trait, resulting in males with more exaggerated ornamentation and females with a greater preference for this ornamentation in the next generation (17, 157, 158). Another theory, Zahavi's handicap hypothesis, predicts that costly traits are most likely to represent honest signals of quality and are thus preferred in

a population (155, 159). A third theory is the sensory exploitation hypothesis, which proposed that females are most sensitive to exaggerated courtship signals (160, 161).

Flies in the family Tephritidae possess a variety of intricate courtship ornaments and behaviors that have likely resulted from sexual selection. Tephritid courtship displays often include visual, acoustic, and chemical cues (127). It has been proposed that an abundant and homogenous resource distribution, in this case oviposition sites on plants, led to male signaling with pheromones and acoustic displays to attract females, whereas more scare resources lead to male defense of territories and male-male aggression. The first scenario provides females with a greater freedom of mate choice and selects for males that advertise their qualities as mates, often resulting in complex courtship displays (127, 162).

In the case of the goldenrod gall fly (Tephritidae: *Eurosta solidaginis* [Fitch]), adult males perch on the apical tips of goldenrod plants (*Solidago altissima* L.), from which they attempt to attract females. Male *E. solidaginis* courtship behavior is fairly complex and includes visual cues such as rocking the body from side-to side and fanning their patterned wings (149, 150). I recently discovered that males also emit very large quantities of a putative sex pheromone (> 70 μg over 24 hr) that is attractive to female flies (83). In many insect mating systems, especially among Lepidoptera, females release pheromones and males follow the pheromone plume to find mates; however, for some taxa, males are the pheromone producer and females respond. Such taxa include many cerambycid species, the lesser wax moth (*Achroia grisella*) and several tephritids, including *E. solidaginis* (57, 83, 127, 163, 164). The male-produced pheromones of tephritid flies are generally released in quantities orders of magnitude higher than female

emitted pheromones. For example, females of the moth *Helicoverpa zea* emit about 1 ng per hour whereas male *E. solidaginis* flies emits about 3 µg per hour (58, 83, 127).

The putative pheromone blend of *E. solidaginis* is dominated by spiroacetals and one long-chain alcohol. Spiroacetals are a diverse group of bicyclic acetal compounds produced by a wide range of insect taxa. The biological function of many spiroacetals remains unclear, however several insect-produced spiroacetals have been identified as pheromones including aggregation, territorial, epideictic, and sex pheromones (47, 165). A few insect-produced spiroacetals also appear to play a role in defense and have been identified among the contents in venom glands of *Vespula* wasps and the defensive secretion of the rove beetle (*Ontholes murinus*) (47). Spiroacetals are thought to be produced from fatty acid precursors and P450 mono-oxygenase enzymes likely play an important role in their synthesis (47, 165). In addition to *E. solidaginis*, several other tephritid species, including many flies in the genus *Bactrocera*, also produce spiroacetals, which can be male- or female-produced sex attractants (47, 127, 165–167). Tephritid flies that release spiroacetals, specifically *Dacus sp.*, *Bactrocera sp.*, and *E. solidaginis*, all possess rectal pheromone glands where spiroacetals appear to be produced (56, 166–170).

The goals of this study were to characterize the chemical composition and release behavior of the male *E. solidaginis* volatile emission and determine whether production of the emission is a predictor of male quality. Determining that male quality correlates with emission production would align with Zahavi's honest signal/handicap hypothesis and provide support for the prediction that the male *E. solidaginis* emission is under sexual selection. My previous work indicates that tall goldenrod plants eavesdrop on the

emission of male *E. solidaginis* and respond by enhancing their anti-herbivore defenses, presumably reducing *E. solidaginis* larval survival; thus, natural selection should favor males that emit less of the emission (83, 84). Male flies, however, typically emit very large quantities of the emission, perhaps suggesting that emission production is under sexual selection. Here I examined whether male emission production is an honest signal of male fly quality, and thus potentially selected for through female mate choice.

Results

Male *E. solidaginis* emission production during one nychthemeron

Male *E. solidaginis* flies emit on average, more than 70 μg of volatile emission in a twenty-four hour period (83). This emission comprises the two main compounds, (7*S*,5*S*)-7methyl-1,6-dioxaspiro[4.5]decane (57.4 ug $\pm$ 5.2) and 1-nonanol (23.0 μg $\pm$ 2.4), as well as several other spiroacetals including (*E*) and (*Z*) 2-methyl-1,6-dioxaspiro[4.5]decane (3.1 μg $\pm$ 0.29). After surveying emission production by individual males over a twenty-four hour light and dark cycle, I found that release of the male *E. solidaginis* volatile emission corresponds with the light period and that peak production tends to occur between 8:00 and 17:00 (Figure 1, Figure 2), during the previously documented periods of active mating, (9:30-16:45; (149, 150). During the dark period, male flies emit little to none of the emission (Figure 1, Figure 2). (Friedman rank sum test *maxT*=4.264, *P*=0.00005; T1-T2 *P*=0.29, T1-T3 *P*=0.00007, T2-T3 *P*=0.02).

Variation in emission production among *E. soldaginis* males and correlation of emission production with traits of male quality

To determine for *E. solidaginis* if there was a positive association between male quality and emission release, I measured traits associated with male quality and related them to the amount of emission each male produced. For measurements of male quality, I found no correlation between mass of *E. solidaginis* males and the mass of the gall from which they emerged, and only a weak correlation between male mass and gall diameter (Simple linear regression for all correlations $F_{1,83}$=2.09, P=0.15, R^2=0.03; $F_{1,83}$=5.46, P=0.022, R^2=0.07; Figure 3). I also detected no relationship between male mass and male lifespan ($F_{1,82}$=0.97, P=0.33, R^2=0.01).

Male emission production was highly variable among males, with individuals releasing between 18.4 and 294.7 μg over 24 hr. Perhaps as a result, I found no correlation between male mass and emission production on either day 2 or day 5 after emergence (Day 2: $F_{1,77}$=0.474, P=0.49, R^2=0.006; Day 5: $F_{1,60}$=0.74, P=0.39, R^2=0.02; Figure 4, Figure 5). I also found no relationship between male mass and the first or second most abundant compounds in the emission blend on both day 2 and day 5 (Compound 1, Day 2: $F_{1,77}$=0.29, P=0.59, R^2=0.0009; Compound 2, Day 2: $F_{1,77}$=0.92, P =0.34, R^2=0.005; Compound 1, Day 5: $F_{1,60}$=1.03, P =0.31, R^2=0.004; Compound 2, Day 5: $F_{1,60}$=0.69, P =0.20, R^2=0.002). Total emission released on day 2 or day 5 was not correlated with male lifespan (Day 2: $F_{1,77}$= 0.59 P=0.44, R^2=0.001; Day 5: $F_{1,60}$= 2.92, P=0.09, R^2=0.05). Neither gall diameter nor mass correlated significantly with pheromone production on day 2 or day 5 (Gall Diameter, Emission Day 2: $F_{1,77}$=0.26, P=0.61, R^2= 0.01; Gall Mass, Emission Day 2: $F_{1,77}$=0.03 , P=0.86, R^2= 0.003; Gall

Diameter, Emission Day 5: $F_{1,60}$=0.48, P=0.49, R^2= 0.009; Gall Mass, Emission Day 5: $F_{1,60}$=0.001, P=0.97, R^2= 0.004).

On average, male *Eurosta* released similar amounts of total emission on day 2 and day 5 (Paired t-test t_{53}=0.70, P=0.49). However, they emitted more of the most abundant compound ((7*S*,5*S*)-7methyl-1,6-dioxaspiro[4.5]decane) on day 5 compared to day 2 (Paired t-test t_{53}=1.71, P=0.09). They also emitted significantly less of the second most abundant compound (1-nonanol) on day 5 compared to day 2 (Paired t-test t_{52}=1.94, P=0.05). The ratio of compound 1:compound 2 increased significantly on day 5 compared to day 2 (Paired t-test, t_{56}=-3.69, P=0.0001; Figure 6). The amount of the third most abundant compound (likely (*E*) 2-methyl-1,6-dioxaspiro[4.5]decane) did not change from day 2 to day 5 (Paired t-test t_{53}=-1.53, P=0.13). The ratio of compound 1:compound 3 (the third most abundant compound) did not change (Paired t-test t_{52}=1.29, P=0.6).

In summary, male *E. solidaginis* emission release was not a good indicator of the traits of male quality that I measured and my findings do not support Zahavi's handicap/honest signal hypothesis. Notably, the blend of the emission changed quantitatively as the flies aged, with older flies emitting more of the most abundant compound relative to the second most abundant compound.

E. solidaginis **rectal gland**

I found that male *E. solidaginis* possess a rectal gland similar to that described for other tephritid species (Figure 7, Figure 8; 21–26). The contents of the gland match the dominant compounds emitted in the volatile blend of *E. solidaginis* and include (7*S*,5*S*)-7methyl-1,6-dioxaspiro[4.5]decane, 1-nonanol, and (*E*) and (*Z*) 2-methyl-1,6-

dioxaspiro[4.5]decane. This indicates that the *E. solidaginis* emission is stored and possibly synthesized in the rectal gland.

Emission from female *E. solidaginis*

Female *E. solidaginis* do not have a characteristic odor and do not emit large quantities of a volatile emission as males do. Some females emit small quantities of (7*S*,5*S*)-7methyl-1,6-dioxaspiro[4.5]decane and 1-nonanol. Two of eight females surveyed emitted 84 ng or 246 ng of (7*S*,5*S*)--7methyl-1,6-dioxaspiro[4.5]decane in 24 hours. Four of eight females surveyed emitted 415 ng, 522 ng, 137 ng, or 380 ng of 1-nonanol. This confirms that females occasionally emit one or two compounds found in the male blend in very low amounts, but I did not explore the role of these compounds for female flies.

Discussion

I found that male *E. solidaginis* release their volatile emission primarily during daylight hours with little to no release during the evening (Figure 1, Figure 2). Previous studies documented that *E. solidaginis* flies are diurnal and actively mating during the warmest part of the day (9:30-16:45; (149, 150). This observation corresponds with the peak release times I documented for the putative male-produced sex pheromone and supports my hypothesis that the emission is involved in female attraction and courtship.

A previous study with the parasitoid wasp *Nasonia vitripennis* found that male pheromone production was positively correlated with sperm production, and thus male

mate quality (171). Because I previously observed high variation among males in emission release, I predicted that *E. solidaginis* emission release would similarly be correlated with traits related to male quality. To assess male quality, I measured male mass, gall mass, gall diameter, and male longevity, but none of these variables were associated with emission production, indicating that this trait was not a good predictor of the male-quality traits that I measured. Perhaps other features of male quality, like sperm production or the quality of the host plant they choose, are associated with production of their volatile emission, but at this point I cannot conclude that the putative pheromone provides female flies with an honest signal for assessing the quality of potential mates.

Gall size, especially gall diameter, is heritable for *E. solidaginis* and is an important trait for larval survival. Classic evolutionary ecology research revealed that *E. solidaginis* gall size is under stabilizing selection for intermediate-sized galls. Small-diameter galls are more vulnerable to attack by the parasitoid *Eurytoma gigantea*, which oviposits into fully developed galls (beginning late June through early August) and parasitism success is limited by ovipositor length (150, 172). In contrast, larvae in large-diameter galls are more frequently attacked and eaten by avian predators such as downy woodpeckers (*Picoides pubescens*) and black-capped chickadees (*Poecile atricapillus*) (150, 173). As a result, *E. solidaginis* larvae in intermediate-sized galls have the highest probability of survival to adulthood (150, 172–174). This stabilizing selection for gall size may help explain why this gall trait is only weakly correlated with adult male mass and is not correlated with male emission release. Perhaps selection for intermediate gall size has limited variation among *E. solidaginis* galls, possibly disrupting the expected relationship (Figure 3).

I also measured male mass as a proxy for male quality because this trait has been found to play an important role in mating success and increased fitness in several insect species, including another tephritid species. Large male size and a protein-rich diet have a positive influence on mating success of male Mediterranean fruit fly, *Ceratitis capitata* (175, 176). Similarly, large male *Drosophila sp.* won aggressive encounters and were most often observed mating (177). Higher rates of mating success for larger males have also been observed in non-dipteran species (178–180). For instance, in absence of competition, females field crickets (*Gryllus sp*.) preferred larger males, which had higher lifetime reproductive success than smaller males (178). However, for male *E. solidaginis*, body size did not correlate with emission release, as I had hypothesized, indicating that pheromone release is more nuanced than just a call for female flies (Figure 4, Figure 5).

The final trait I measured was adult lifespan. I predicted that higher quality *E. solidaginis* males would have a longer lifespan and the longer lifespan could increase a male's chance of mating successfully. I did not observe a correlation between male emission release and lifespan indicating that emission release is neither a signal of male longevity nor imposes a measurable cost for male survival.

In summary, male *E. solidaginis* emission release was not correlated with any of the traits representing male quality that I measured. It is possible that other traits related to male quality might be significantly correlated with male emission release, such as symmetry, ability to manipulate gall nutrition or defense, or selection of a suitable host plant. Fluctuating asymmetry can be an indicator of environmental stress during development, is sometimes heritable, and often correlates negatively with protein heterozygosity and individual fitness. Previous studies have documented symmetry as a

preferred trait for females, for example Japanese scorpion flies (*Panorpa japonica*) and field crickets (*Gryllus campestris*), during mate choice (181, 182). It is possible that male *E. solidaginis* symmetry is an important indicator of male quality and future work should examine the correlation of male symmetry and emission production. Gall manipulation for reduced anti-herbivore defenses or increased nutrition could also be important for male quality and should also be assessed. Previous work has documented *E. solidaginis* ability to attenuate host plant defenses and manipulation resource allocation (150, 183). Additionally, male *E. solidaginis* select *S. altissima* plants as perch sites from which to signal and attract females. It is possible that males who choose more preferred host plants are also of higher quality, which could be reflected in their emission release.

Based on my findings to date, it does not appear that male *E. solidaginis* emission release is not an honest signal of male quality, at least for the traits I measured, and thus cannot be explained with Zahavi's honest signal/handicap hypothesis. An alternative hypothesis related to male signaling and sexual selection states that female behavior might evolve because it enables them to find a mate, but does not necessarily allow them to discriminate among individual suitors. In this case, selection could favor exaggerated male calling because selection has been for females to minimize the time and difficulty required to find a mate (11, 158). Alternatively, signals might evolve to exploit sensory biases in their receivers, in this case leading to male *E. solidaginis* that emit larger quantities of the emission to attract receptive females (160). An important future study would be to assess evidence for additional hypothesis related to sexual selection, especially female preference for *E. solidaginis* emission blends and concentrations.

Notably, I found that as male *E. solidaginis* flies age, the blend of their volatile emission changes and they emit more (5S,7S)-7-methyl-1,6-dioxasopiro[4.5]decane compared to younger males and less 1-nonanol (Chapter 4). This emphasizes the possibility of conflicting selection pressures exerted on male *E. solidaginis* emission production from *S. altissima* plants and female *E. soldaginis*. If (5S,7S)-7-methyl-1,6-dioxasopiro[4.5]decane is the active compound in the blend responsible for priming goldenrod defenses, selection should favor male *E. solidaginis* that emit less of this compound as their larvae would have a better chance at survival. However, if female *E. solidaginis* favor males who emit larger amounts of (5*S*,7*S*)-7-methyl-1,6-dioxasopiro[4.5]decane, these males would have a better chance of mating and leaving offspring. It is therefore particularly interesting that virgin male *E. solidaginis* emit larger amounts of (5*S*,7*S*)-7-methyl-1,6-dioxasopiro[4.5]decane as they age. This may represent a strategy for males to emit less of the emission as they are young, thus limiting cues for *S. altissima* eavesdropping, and to emit larger quantities of (5*S*,7*S*)-7-methyl-1,6-dioxasopiro[4.5]decane when they are older to improve their chance of mating before they die. This change in ratio also provides a signal for female *E. solidaginis* to potentially assess male age. Previous work has observed that older males are less preferred and likely to mate than younger males, which is possibly explained by our findings (150).

Finally, I also observed that virgin female *E. solidaginis* emit small quantities of one or more of the compounds in the male volatile emission. Because no females emitted the full blend of compounds or large quantities of any of the compounds, it appears that the *E. solidaginis* volatile emission is specific to male flies. However, because these

females were kept in isolation it appears that they must share some of the same biosynthetic machinery as male *E. solidaginis.* Future study is needed to dissect female *E. solidaignis* to determine whether they possess any form of rectal glands or the associated organs.

Materials and Methods

The study system

In spring, *E. solidaginis* larvae break their winter diapause and pupate, with adult flies typically emerging around mid-May in Pennsylvania. Male *E. solidaginis* perch on *S. altissima* plants where they emit large quantities of a putative sex pheromone that is attractive to female flies (mean ~70 ± 20 μg 24 h^{-1}; 14). Mated female flies select favorable *S. altissima* plants and oviposit into the apical buds, with eggs typically hatching around one week later. The developing *E. solidaginis* larvae induce sphere-shaped galls on the stems of the plants that become visible within approximately three weeks of oviposition (149, 150).

Male *E. solidaginis* emission release during one nychthemeron

To determine what time of day male *E. solidaginis* flies release emission, I collected and analyzed emission from individual male flies during three-hour intervals over the course of a twenty-four hour light-dark cycle. To obtain flies for the analysis, I collected *E. solidaginis* galls near State College, PA, USA in February and March of 2014 and 2015. Galls were stored in a freezer at -20°C to maintain larval diapause and

then transferred to room temperature, approximately 22°C, to induce pupation and emergence of adult flies. Galls were placed into individual cups to ensure isolation of individuals upon emergence. I misted the galls with water two times per week to prevent desiccation. The adult *E. solidaginis* flies emerged between twenty-one and thirty days after removal from the freezer.

After they emerged, I placed virgin male flies into individual glass chambers containing a clean, moist piece of cotton. I collected emission from the male flies for three-hour intervals over a total of twenty-four hours. The intervals were as such: T1=5:00-8:00, T2=8:10-11:10, T3=11:20-14:20, T4= 14:30-17:30, T5= 17:40-20:40, T6= 20:50-23:50, T7=0:00-3:00, T8=3:10-6:10. Using a push/pull volatile collection system, filtered air was pushed into each chamber at a rate of 0.6 L per minute and pulled out of the chamber, over a clean adsorbent filter containing Super-Q (Alltech), at a rate of 0.5 L per minute. A separate, clean filter was used for each collection interval. The growth chamber used for collections was set to a 18:6 light-dark cycle with the photophase from 6:00 to 22:00 and the scotophase from 22:00 to 6:00. The temperature was 22°C during the light period and 20°C during the dark period and the relative humidity was 65%. After collecting emission, I eluted each filter using 150 µL dichloromethane. I added 5µL of a standard solution containing 200 ng n-octane and 400 ng nonyl acetate to each sample and analyzed these samples using GC-FID. Samples were injected in 1 µL aliquots into an Agilent Technologies model 7890A gas chromatograph fitted with a flame ionization detector (column: Agilent HP-5 25 µm x 320 µm x 30 m). The column was held at 40°C for 1 min then increased by 20°C per minute to 300°C. I calculated the quantities of the emission components emitted by each fly using MSD Chemstation

(Agilent Technologies) by measuring volatile output in nanograms relative to the internal standard. Compounds were identified using coupled GC-MS in collaboration with Hans Alborn (US Department of Agriculture, Gainesville, FL, USA) and Wittko Francke (University of Hamburg, Hamburg, Germany).

The total emission produced during each three-hour interval for individual *E. soldiaginis* male was calculated by taking the sum of the three most dominant compounds in the blend. The three compounds were identified as (7*S*,5*S*)-7methyl-1,6-dioxaspiro[4.5]decane, 1-nonanol, and likely (*E*) 2-methyl-1,6-dioxaspiro[4.5]decane. The total emission produced by each male was plotted for each interval.

I calculated the total emission produced during each three-hour interval for individual *E. soldiaginis* males by taking the sum of the three most dominant compounds in the blend. The three most abundant compounds in the blend were identified as 1) ((7*S*,5*S*)-7methyl-1,6-dioxaspiro[4.5]decane, 2) 1-nonanol, and 3) likely (*E*) 2-methyl-1,6-dioxaspiro[4.5]decane. I plotted the total emission produced by each male for each interval in the twenty-four hour period. I also standardized the emission production for individual males during each interval by dividing the interval total by the total for the twenty-four hour period and then plotted these values. To compare emission production during the light and dark periods, I pooled adjacent time intervals to create one six-hour dark interval and two six-hour light intervals. The two time intervals containing hours of both light and dark were excluded from the analysis. The new time intervals were T1=8:10-14:20, T2= 14:30-20:40, T3=0:00-6:10. I analyzed the difference in emission production among the three intervals using a Friedman's Test for non-parametric randomized block analysis of variance. This test was selected as a non-parametric

alternative for a one-way ANOVA with repeated measures because the data do not meet the assumptions of equal variances and the sample size is too small to assume a normal distribution.

Variation in emission production among *E. soldaginis* males and correlation of emission production with with traits of male quality

To determine the amount of variation in emission production among *E. solidaginis* males and whether emission production is correlated with male quality, I collected emission from individual males and examined traits I predicted could indicate male quality.

To obtain flies for the analysis, I collected galls near State College, PA, USA in February and March 2014. The galls were stored in a freezer at -20°C to maintain larval diapause and then transferred to room temperature, approximately 22°C, to induce pupation and eclosure from the gall. To ensure broad sampling of plant and fly genotypes and gall sizes, I collected galls by walking transects through galled goldenrod habitat and collecting all galls detected that were larger than approximately 1 cm in diameter. Galls with visible holes from parasitoids and avian predation as well as stems where multiple galls were not differentiated were avoided.

In the lab, the stems were trimmed from the top and bottom of the gall, flush with the round part of the gall. I weighed the galls and recorded the diameter. Then I placed each gall in an individual diet cup moistened with water. Adult flies emerged between twenty-one and thirty days after being placed at room temperature. Each day, I removed

the emerged flies, recorded their sex, and weighed them. I removed the galls from the diet cups, added a piece of moist cotton and kept at room temperature.

On the day following the flies' emergence, the male fly emission production was measured. Individual males were placed inside glass collection chambers (2.5 L volume) filtered air was pumped into the chambers at 0.5L/min and was pulled out of the chambers at 0.5L/min over adsorbant filters. A clean, moist cotton ball was also placed inside each chamber to add humidity and reduce dehydration stress for the flies. A foil shade was placed on top of each glass dome, but not completely covering, to shade the flies from direct light and prevent excessive temperatures from building inside the glass domes. The light cycle for the growth chamber was 6:00-22:00 light, 22:00-6:00 dark; the temperature was 22°C day and 20°C night; and the relative humidity was 65%. Each collection period was from 9:30 to 16:30, which corresponds with my previous work demonstrating peak emission release during these hours as well as previous reports that flies are actively mating during these hours (149).

Following this collection, I removed the flies from the chambers, weighed them, and returned them to their individual cups. On the fourth day following emergence, I again weighed each fly and returned him to a glass collection chamber. Following the procedure described above, I collected emission on the fifth day after emergence, weighed them and returned them to their cups. Flies were then kept at room temperature with a moist cotton ball until they died to record the adult lifespan.

I examined possible correlations among the traits I measured using simple linear regression models. A paired t-test was used to compare the ratio of compound 1:compound 2 on days 2 and 5.

E. solidaginis rectal gland

To determine what type of gland *E. solidaginis* males possess and whether the contents of the gland match the fly volatile emission, I dissected male flies and located the rectal gland. I extracted the glands in dichloromethane and analyzed the contents using GC-MS.

Emission from female *E. solidaginis*

To determine whether female *E. solidaginis* emit the volatile emission, I aerated individual females and analyzed their odors. I obtained virgin female *E. solidaginis* following the same procedure used for male flies by collecting galls near State College, PA, USA and allowing the adult flies to eclose. I then collected female-produced volatiles for 24 hr and analyzed them following the same procedure described above.

Acknowledgements

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Figures

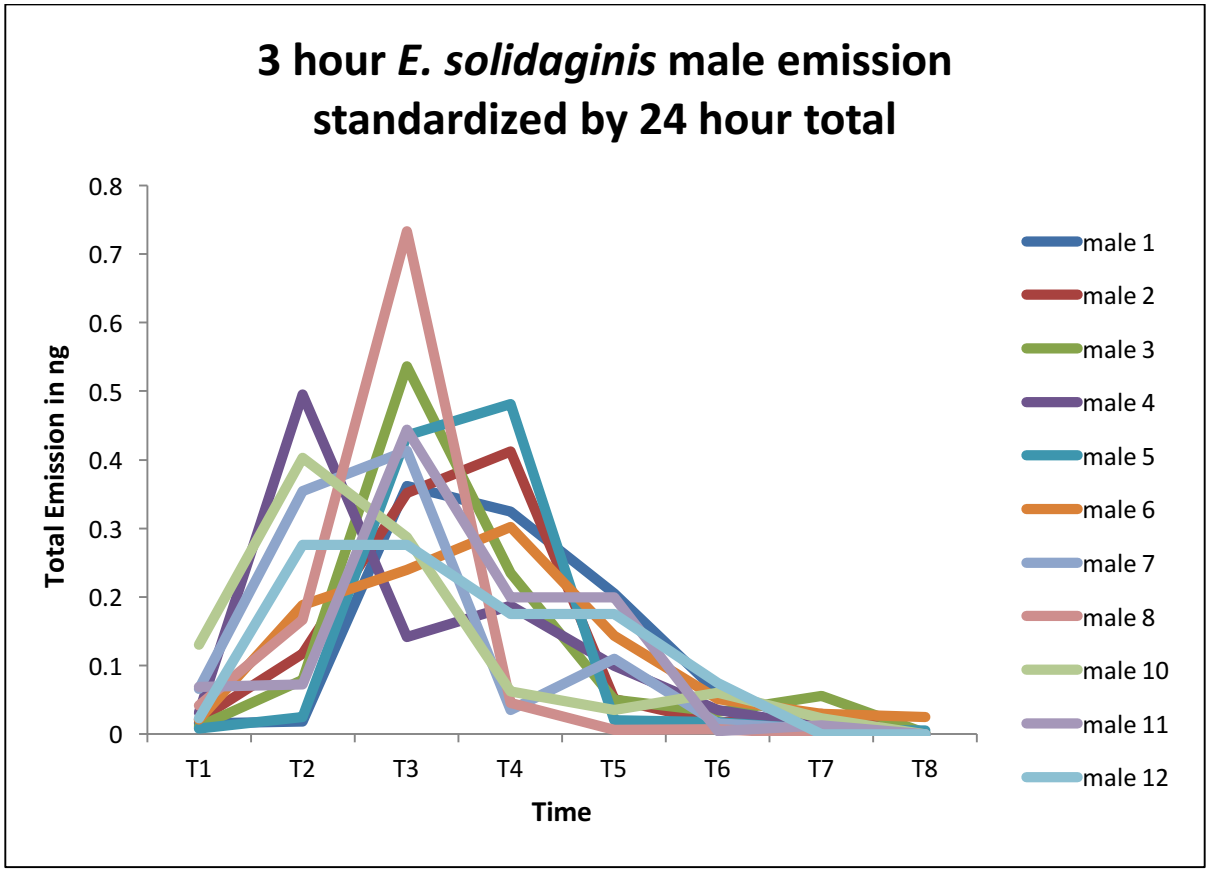
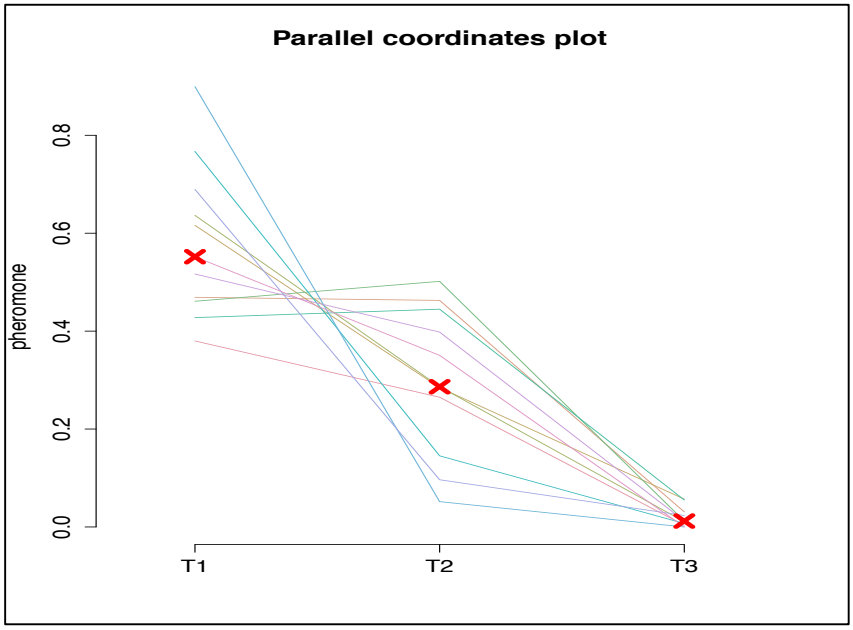


Figure 5-1
Total emission production from individual male *E. solidaginis* during three-hour intervals over the course of twenty-four hours. Values were standardized by the total emitted during the twenty-four hour period. (T1 = 5:00-8:00, T2 = 8:10-11:10, T3 = 11:20-14:20, T4 = 14:30-17:30, T5 = 17:40-20:40, T6 = 20:50-23:50, T7 = 0:00-3:00, T8 = 3:10-6:10.)



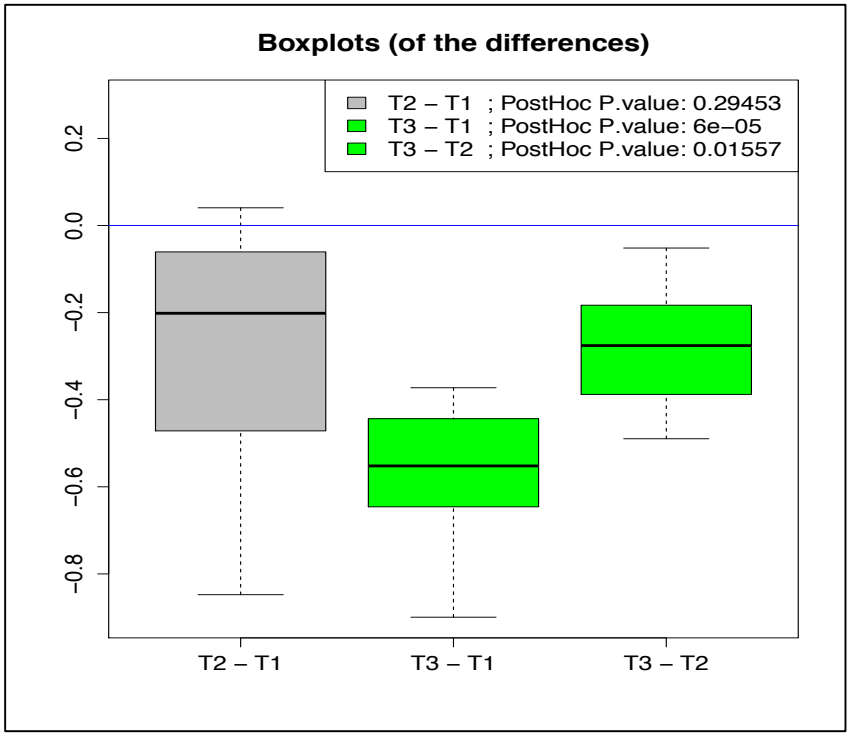


Figure 5-2 A,B
(A.) Total emission produced during six-hour intervals representing light and dark periods. X represents the standardized mean. (B.) Male *E. solidaginis* released significantly more emission during the light period than the dark period T1=8:10-14:20, T2= 14:30-20:40, T3=0:00-6:10

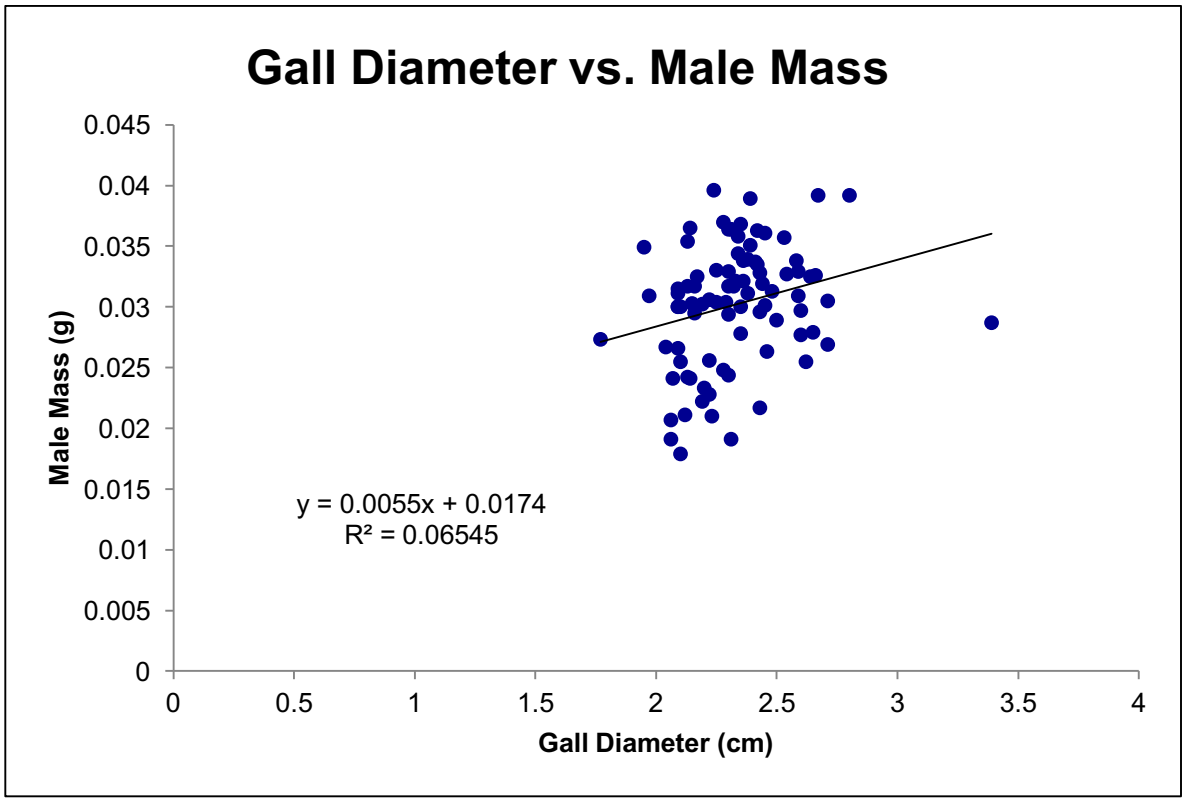


Figure 5-3
Scatterplot of *E. solidaginis* gall diameter and male mass

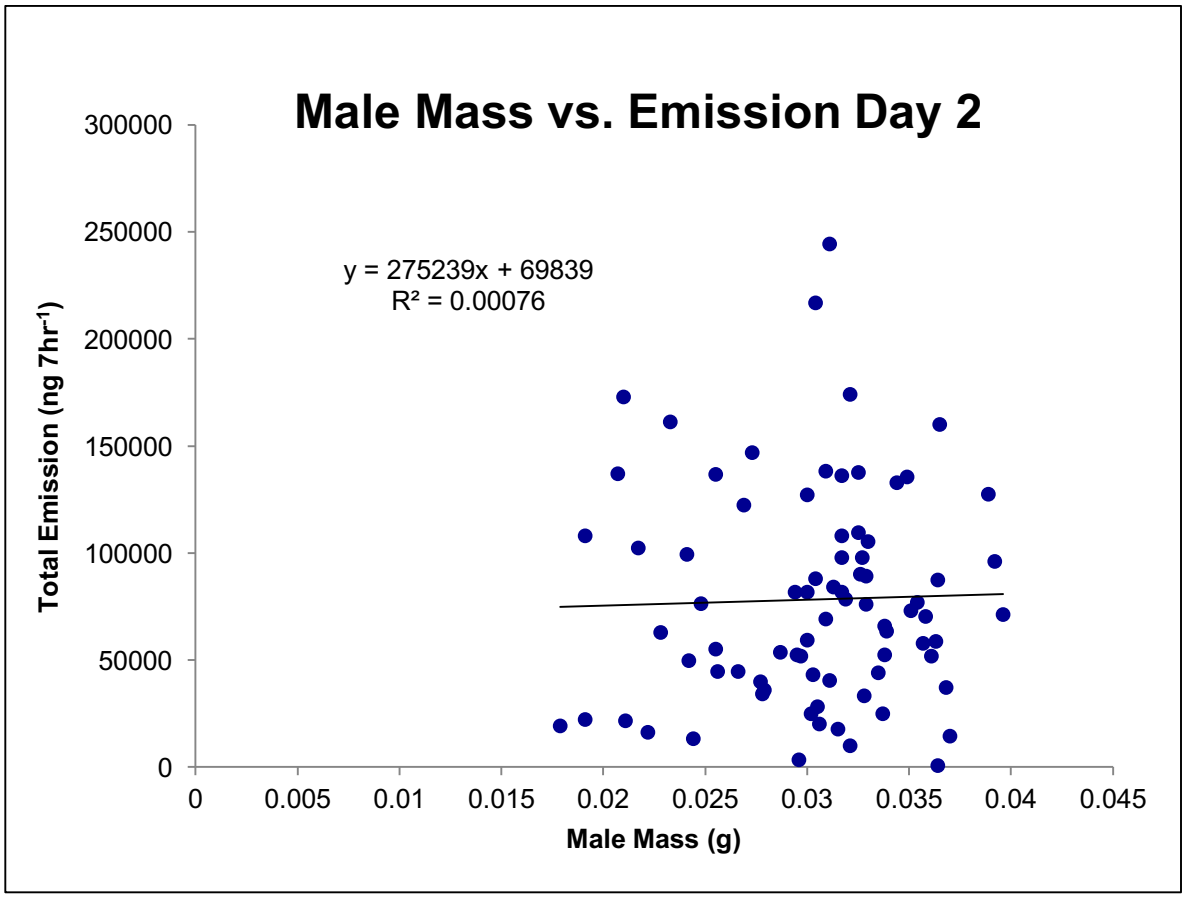


Figure 5-4
Scatterplot of *E. solidaginis* male mass and total emission released on the second day after adult emergence.

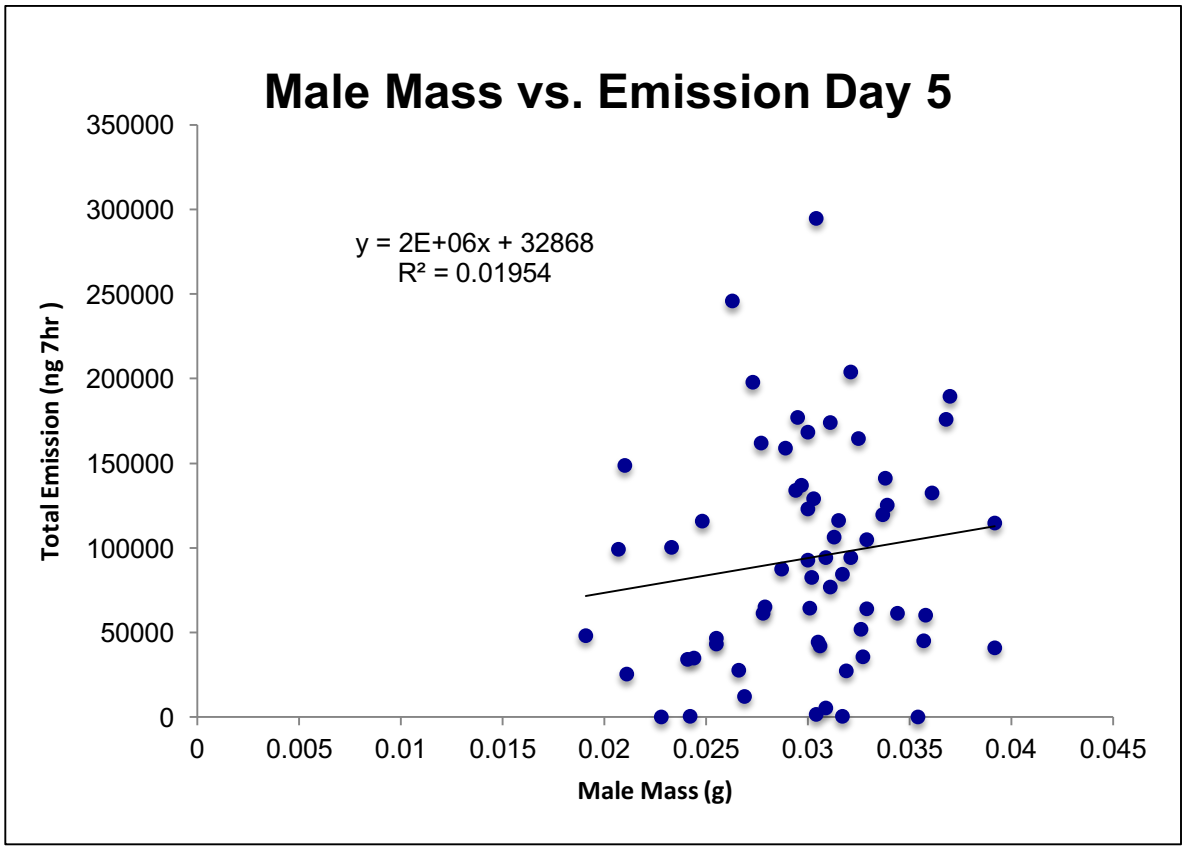


Figure 5-5
Scatterplot of *E. solidaginis* male mass and total emission released on the fifth day after adult emergence.

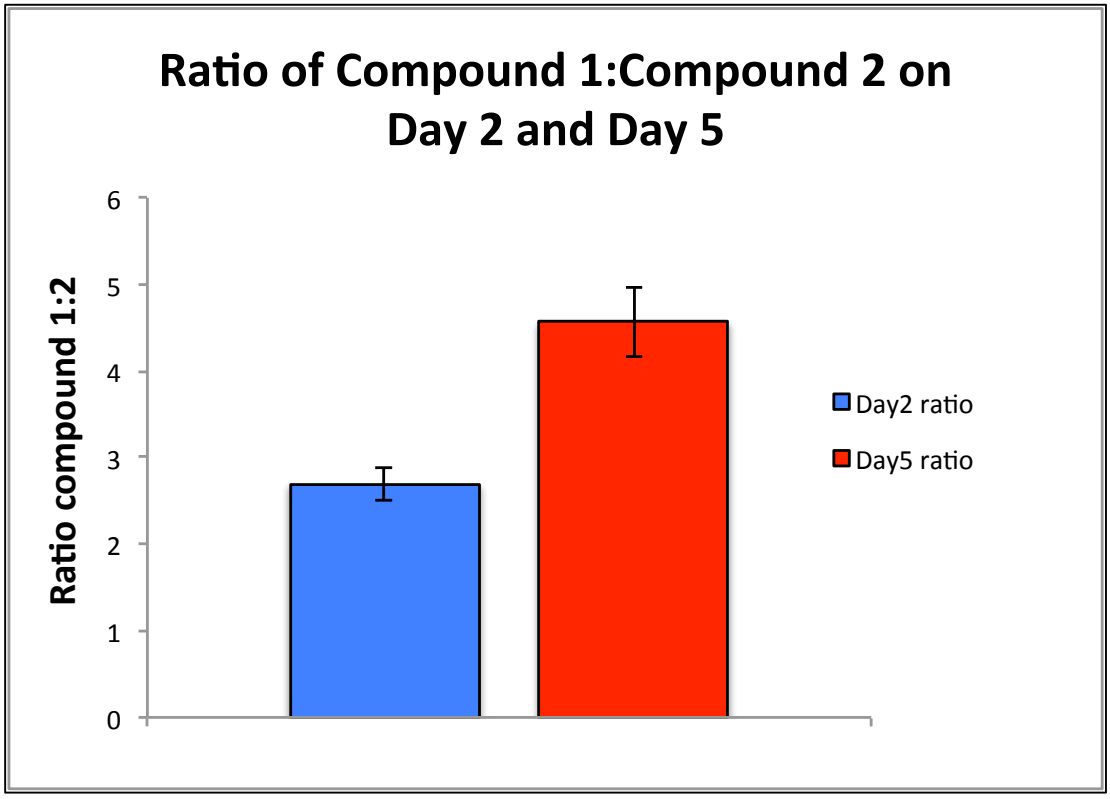


Figure 5-6
Ratio of most abundant compound ((7*S*,5*S*)-7methyl-1,6-dioxaspiro[4.5]decane) and the second most abundant compound (1-nonanol). The ratio of compound 1:compound 2 was significantly greater for five-day-old flies than for two-day-old flies (Paired t-test, t_{56}=-3.69, P=0.0001).

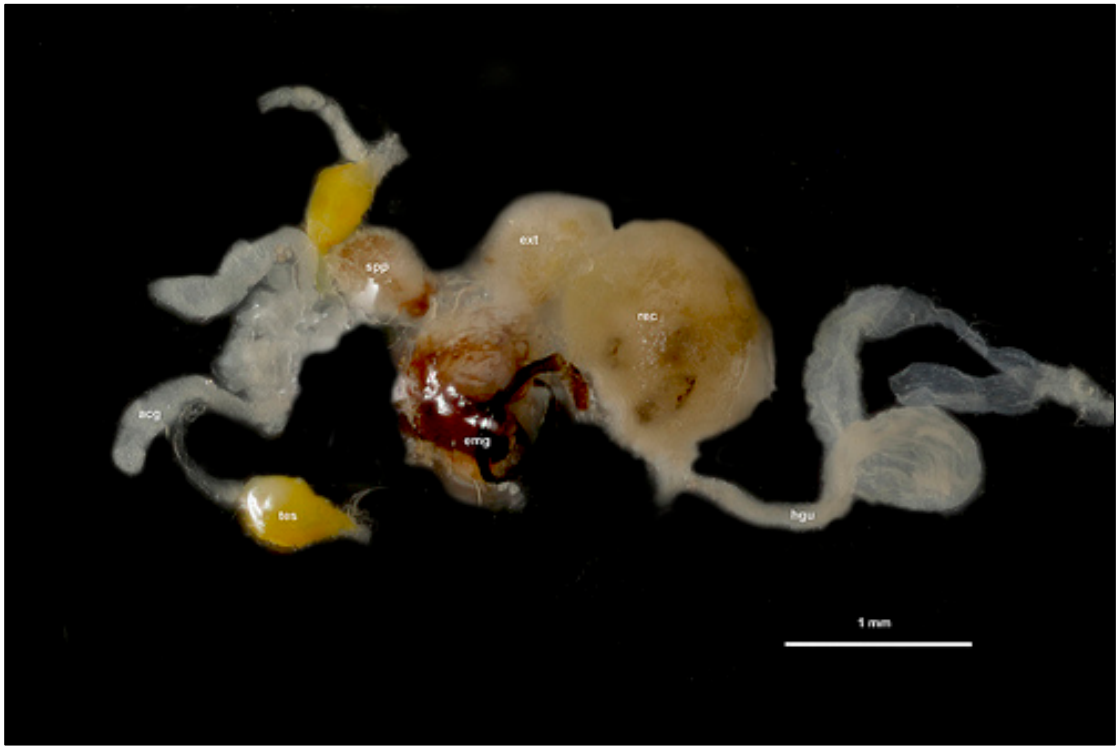


Figure 5-7
Rectal Gland
Bright field image of *Eurosta solidaginis* showing the hindgut and male terminalia. Note that the specimen has emptied the rectal gland (rec) during the dissection so it is reduced comparing to its original "filled" size. tes=testis, spp=sperm pump, acg=accessory gland, emg=external male genbitalia, ext=secretory sac, rec=rectal gland, hgu=hind gut. Photo by István Mikó (CC BY 2.0)

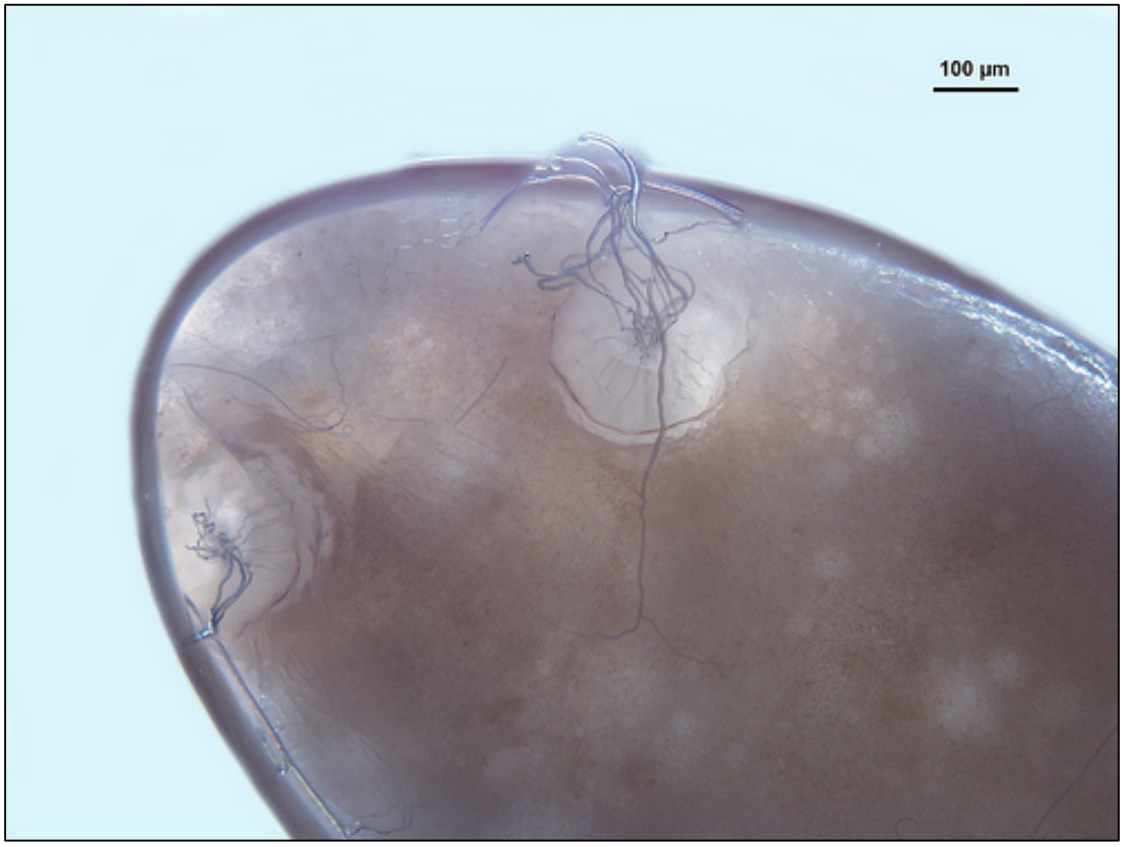


Figure 5-8
Rectal Pad
Bright field image of *Eurosta solidaginis* showing the rectal pads on the “rectal gland”. Photo by István Mikó (CC BY 2.0).

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Chapter 6

Conclusions and future directions

Plants in all natural systems face the threat of being eaten by insects and other herbivores. To protect themselves from their herbivore attackers, plants have evolved a variety of defense strategies, including the production of defensive chemicals. Because these defenses are often costly to produce and maintain, many plants wait to induce or turn on their defenses until they are directly threatened by herbivore damage. It has been well established that insect feeding damage triggers plant defenses, however, more recent discoveries have revealed that plants not only respond to actual feeding damage, but can also tune in to environmental cues associated with the presence of insect herbivores, allowing them to prepare their defenses for future attack. Some of the cues that plants use are physical, for example, insect footsteps on leaves or the presence of insect eggs. Additionally, some plants use olfactory cues to sense the presence of nearby herbivores. Several studies have found that plants can eavesdrop on odors emitted by neighboring plants. Insect-damaged plants emit characteristic blends of volatile compounds. Undamaged plants can perceive these odors and respond by enhancing their own anti-herbivore defenses. Because plants perceive odors from other plants to detect herbivores, I hypothesized that they might also perceive odors emitted directly by the herbivores, a possibility that has not been previously considered. Many insect herbivores communicate using pheromones, and often emit these chemical signals in proximity to their host plants.

Therefore, for my dissertation research, I tested whether plants can eavesdrop on insect pheromones to gain a reliable cue of future herbivore damage.

I predicted that an insect pheromone would most likely provide a reliable cue of future herbivory in a system where a specialist herbivore has co-evolved with a single host-plant species. Because of their long co-evolutionary history, my study focused on the plant, tall goldenrod (*Solidago altissima*), and its specialist, gall-inducing fly, *Eurosta solidaginis*. In the spring of each year, male *E. solidaginis* flies perch on the tops of goldenrod plants and emit very large quantities of a putative sex pheromone to attract mates. After mating, the female fly lays her eggs in the stem of the goldenrod plant, where the developing larvae induce ball-shaped galls. These galls have been found to reduce the reproductive fitness of their host plants, indicating that goldenrod would benefit from the ability to detect the presence of the flies and induce effective defenses against the developing larvae.

In the second chapter, I tested the hypothesis that goldenrod plants can perceive the putative *E. solidaginis* pheromone and respond by enhancing their anti-herbivore defenses. I conducted field and laboratory experiments examining how the fly emission influences the defense responses of goldenrod plants. I found that goldenrod plants exposed to the fly pheromone received significantly less herbivore damage than the unexposed control plants. Damage on these plants was reduced from female *E. solidaginis* flies, as well as a suite of different leaf-chewing herbivores, indicating that the exposed plants were less palatable and better defended. Next, I searched for a biochemical signature of this enhanced defense response. For this experiment, I first

exposed plants to the fly emission or a solvent control and then measured changes in the plants' defense chemistry while herbivores were feeding. I found that emission-exposed plants induced substantially higher amounts of jasmonic acid, a key defense phytohormone following herbivore damage. Taken together, these results provide strong evidence that goldenrod plants perceived the *Eurosta* pheromone and responded to it by enhancing their anti-herbivore defenses.

In the third chapter, I tested the hypothesis that the *E. solidaginis* emission enhances *S. altissima* defense responses and does not (directly) deter herbivore feeding. I conducted growth chamber experiments to examine the influence of the *E. solidaginis* emission on *S. altissima* herbivore-induced volatile production. I also conducted greenhouse feeding experiments to determine whether the *E. solidaginis* emission directly deters herbivore feeding on *Cucurbita pepo* or *Symphyotrichum lateriflorum* plants—which have no co-evolutionary relationship with *E. solidaginis* and thus are not expected to exhibit priming responses to the fly emission. I found that *S. altissima* plants with previous exposure to the fly emission exhibited stronger induction of herbivore-induced plant volatiles (HIPV) relative to unexposed controls. I did not observe a similar effect in *E. solidaginis* emission-exposed maize plants (*Zea mays*), which have no co-evolutionary relationship with *E. solidaginis*. I also found no difference in insect feeding damage on *E. solidaginis* emission-exposed *C. pepo* or *S. lateriflorum*. These results provide additional evidence that *S. altissima* plants enhance their anti-herbivore defenses following exposure to the *E. solidaginis* emission and also demonstrate that the emission does not directly deter insect herbivory.

Because the mechanism of how plants perceive olfactory cues and the specificity of plant olfaction are poorly understood, in the fourth chapter, I also investigated goldenrod responses to the individual chemical constituents of the *E. solidaginis* emission blend. The *E. solidaginis* emission comprises two major chemical compounds, the spiroacetal (*7S,5*S)-7-methyl-1,6-dioxaspiro[4.5]decane, and the long-chain alcohol 1-nonanol. The emission also contains small quantities of several other spiroacetal compounds that include (*E*) and (*Z*) 2-methyl-1,6-dioxaspiro[4.5]decane. My goal in this study was to determine if goldenrod plants perceive either of the major compounds or the spiroacetals (*E*) and (*Z*) 2-methyl-1,6-dioxaspiro[4.5]decane. To test this, I exposed some plants to a single compound from the blend and others to the entire emission blend or a solvent control. I found that goldenrod plants exposed to the most abundant compound in the blend, (*7S,5*S)-7-methyl-1,6-dioxaspiro[4.5]decane, received less herbivore damage and induced higher quantities of defensive chemicals than control plants, a similar result to the plants exposed to the complete blend. However, the second most abundant compound in the *E. solidaginis* emission, 1-nonanol, did not enhance *S. altissima* defenses or reduce herbivore damage. (*E*) and (*Z*) 2-methyl-1,6-dioxaspiro[4.5]decane appeared to induce an intermediate response that was not statistically different from any other treatment. These findings indicate that the most abundant compound in the emission is important for goldenrod plants to eavesdrop on *E. solidaginis* flies. This result provides some insight into the specificity of *S. altissima* olfactory perception and suggests goldenrod plants may possess a fairly specific receptor, bringing us one step closer to resolving the mechanism of how plants can smell.

Through this research, I also found that male *E. solidaginis* flies emit very large quantities of a putative sex pheromone (~70 ± 20 µg over 24 h). The finding that goldenrod plants eavesdrop on the *E. solidaginis* emission, thereby reducing host-plant quality and potentially fly fitness, led me to the questions "Why do male *E. solidaginis* flies produce so much emission?". Organisms are often under sexual selection to express otherwise expensive or costly traits that their mates find attractive, to increase their likelihood of reproducing. I hypothesized that *E. solidaginis* males are under sexual selection to emit large quantities of emission despite the cost of providing a cue for *S. altissima* plants to enhance their defenses and subsequently reduce larval performance. To help answer this question, I measured emission release as well as other physical characteristics of individual male flies, such as mass, gall-size, and life span to determine whether production is correlated with male quality. I found that emission release is not correlated with gall size, male mass, or life span. This indicates that male emission production is not an honest signal of male fly quality based on the traits I measured. This provides some support for the hypothesis that high emission production is under selection by female flies. I also found that the ratio of compounds in the male emission blend changes with fly age. This change in the emission blend may provide female *E. soldidaginis* with an honest signal of male age and may even influence the efficiency of goldenrod eavesdropping.

The findings from my dissertation research have revealed an entirely new class of volatile-mediated interactions between plants and insects as this is the first time anyone has shown that plants can perceive animal-derived odors. My dissertation research

documents the first known example of plants perceiving insect odors and demonstrates that a single compound is sufficient to elicit a response in goldenrod plants. This work has important implications for the evolutionary ecology of plant-insect interactions as it reveals a previously unknown level of complexity in signaling between plants and insects. From these results, we can conclude that olfactory communication in plants occurs more frequently than previously thought and that plants are incredible opportunists that may take advantage of a wide variety of environmental cues to help them reliably predict impending herbivory. These results also reveal that insect pheromone communication might be subject to selection pressure in the co-evolutionary arms race between plants and insect herbivores.

One of the key unanswered questions related to my dissertation research is the mechanism of how plants perceive odor cues. The *S. altissima* and *E. solidaginis* study system could provide a unique opportunity for exploring the mechanisms of plant olfaction and enhancing plant defenses, which remain poorly understood. My findings demonstrate that *S. altissima* plants perceive the most abundant compound in the *E. soldiaginis* emission and not the second most abundant compound, indicating some level of specificity. Only a few other studies to date have attempted to track plant perception of specific volatile compounds and these studies have focused exclusively on plant-produced odors. To get a better sense of the specificity of *S. altissima* olfactory perception, it would be beneficial to test *S. altissima* responses to more individual compounds, especially other spiroacetals. This would allow us to determine whether *S. altissima* plants are generally responsive to spiroacetal compounds or more specifically

respond to the most abundant compound in the *E. solidaginis* emission. To begin searching for a mechanism of plant olfactory perception, one could identify unresponsive *S. altissima* genotypes and compare their differences in gene expression before and after exposure to the *E. solidaginis* emission.

Another unanswered question from my dissertation research is whether the *E. solidaginis* emission is truly under sexual selection by female *E. solidaginis*. To answer this question, one could perform behavioral assays with female *E. solidaginis* to determine if which emission concentration or particular blend ratios are most attractive to the female flies. Additionally, one could measure other potential traits of male quality for *E. solidaginis* males such as body symmetry, the suitability of the host plant selected for calling, or the volume of sperm produced. In my previous work it is possible that I did not capture an important correlation of emission production with male quality.

VITA

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EDUCATIONAL BACKGROUND

Ph.D. Ecology (2015) Department of Entomology, Pennsylvania State University. Graduate Minor in Statistics. Advised by Dr. John F. Tooker and Dr. Mark C. Mescher.

B.S. Biology, with honors, B.A. Biochemistry (2009) Natural Science Division, Pepperdine University. Minor in German Language. Advised by Dr. Stephen Davis.

PUBLISHED MANUSCRIPTS

Ray, S., Gaffor, I., **Helms, A.M**., Chuang, W., Tooker, J.F., Felton, G.W., and Luthe, D. (2015) Fecal contamination of food increases herbivore performance on plants. (accepted at the ***Journal of Chemical Ecology***)

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GRANTS, AWARDS, AND FELLOWSHIPS

Penn State University Alumni Association Dissertation Award (2015)
Penn State University Michael E. Duke Memorial Award (2014)
President's Prize 1st Place Presentation Entomology Society Student Competition (2012, 2013)
Ralph O. Mumma Outstanding Graduate Student Award (2013)
Best Student Oral Presentation Eastern Branch Entomology Society Meeting (2013)
NSF Graduate Research Fellowship (2011 - 2014)
Pennsylvania State University Graduate Fellowship (2010 - 2011)
Robert W. Graham Endowed Graduate Fellowship (2010 - 2011)
Fulbright Fellowship (2009 - 2010)
DAAD Deutscher Akademischer Austausch Dienst Study Scholarship (2009 declined)