# AN INSECT PHEROMONE CHANGES PLANT BEHAVIOR: LEVERAGING A SHARED SENSORY CUE FOR CROP PROTECTION

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# AN INSECT PHEROMONE CHANGES PLANT BEHAVIOR: LEVERAGING A SHARED SENSORY CUE FOR CROP PROTECTION

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As demand for agricultural products rapidly grows, so too does the demand for effective biocompatible pest treatments. One area for novel treatments that has illustrated promise is the use of semiochemicals to alter the behavior of pest insects. By using these message-carrying compounds, growers can safely attract target insects to traps, repel them from areas under management, or simply confuse them. Currently, the semiochemicals used are typically derived from the pest insects such as pheromones, however insects detect and respond to a diversity of cues. Recent research has shown that the odors of predators can also elicit a strong response in prey, leading to questions about their use to protect crop plants such as: Can the synthetic cues of predators be effectively deployed as a crop treatment? Even broader, semiochemical treatments can also provide important information for other trophic levels in a system such as the plants we are often attempting to protect. This multiple trophic effect highlights the potential of expanding semiochemical treatments beyond just the target pest. Many compounds in the biosphere are shared across trophic levels and this phenomenon may be exploited to both meet management goals and to improve our understanding of the sensory ecology of systems. The overarching goal for this dissertation was to learn how certain compounds affect multiple organisms in an agricultural setting and to develop and test the use of a synthetic predator pheromone treatment to reduce pest damage. To

meet this goal, much had to be learned about the organisms involved and in doing so, unexpected and fascinating discoveries were made along the way.

The study system involved *Solanum tuberosum* (potato), the most damaging insect pest of *S. tuberosum* and other Solanum crops *Leptinotarsa decemlineata* (Say) (Colorado potato beetle), and their widely distributed predator, *Podisus maculiventris* (Say) (spined soldier bug). Building on previous research in this system and after numerous trials, it was established that a large pheromone gland, the dorsal abdominal gland, contained within the predator was eliciting the strongest response in the beetle prey. In the first chapter, I set out to learn more about the development of the dorsal abdominal gland (DAG) and the release behavior of the gland contents. First, *P. maculiventris* were dissected at three adult stages and the chemical composition and amount of each compound was quantified. This revealed that the glands are not formed within 24 hours post eclosure and were mostly formed after seven days. After learning about the physiology of the gland development, the pheromone release behavior was investigated next. By using VOC dataloggers, we found that no pheromone releases were detected in the newly eclosed adults and that the majority of the mature adult volatile chemical releases occurred in a scotophase pattern. These findings add new information about the development of exocrine glands in Pentatomidae and improve our understanding of how odors from the *P. maculiventris* modulate species interactions in cropping systems. The ephemeral properties of the DAG semiochemical also helps explain some of the variability in treatment effect noted in past tests using live *P. maculiventris* as an odor source.

In chapter 2, I field tested the use of live predators as an odor source and compared *L. decemlineata* response to a synthetic dorsal abdominal gland formulation in open dispersion release devices. During this preliminary work of deploying the treatments in the field, a period of time was spent piloting various release devices to find a functional, safe, and economical option to administer the synthetic *P. maculiventris* pheromone. Across two field seasons the results showed that feeding damage by *L. decemlineata* was 22 percent lower in predator odor treated plots, however the effect varied over the season, and in the second year the treatment effect was lost. Interestingly, the synthetic predator pheromone reduced plant damage more consistently than the live predator treatment. These results suggest that temporal patterns of predator cue release and strength may drive the prey's response across the season, and that the synthetic pheromone dispensers may be a viable option to modify pest insect behavior in agricultural systems.

Following the promising field results, in Chapter 3 I set out to better describe the mechanisms behind the prey response in order to optimize the synthetic predator dispensers. Most all organisms enact protective measures to reduce the chance of being consumed. Analogous to the concept of trophic cascade, we have generally considered the cues that are responsible for eliciting anti-predation behavior to follow a downward linear path, where the cues emanating from higher trophic levels cascade downward to prey. However, the role of basal trophic levels such as plants and their own ability to sense and respond to their environment has the potential to influence prey. I used lab and field experiments to ask if the predator pheromone alters plant quality and reduces the performance of *L. decemlineata*. I found evidence that the predator pheromone induces a defensive response in plants and reduces herbivore prey presence at all life stages and the amount the plant material they consume. The results expand our understanding of species interactions by considering the movement of sensory information, where the cues released from an insect predator are perceived by a plant, which has a negative effect on an herbivorous prey species. In light of these findings, we use the term *indirect non-consumptive effects* to describe predator-prey interactions where predatory sensory cues affect prey through another trophic level.

Intrigued by the behavioral changes in the plant that were elicited by the predator pheromone, in Chapter 4 I fractionated the pheromone to better understand the plant response. Of the 5 primary compounds in the pheromone blend, 2 of the compounds are also shared with *S. tuberosum* as green leaf volatiles. Green leaf volatiles are released aerially from nearly all green plants when damaged that are then available for other portions of the plant and neighboring plants to detect and prepare for a potential damage agent via priming of plant defensive metabolites. My hypothesis was that the portion of the predator pheromone that is shared with plant green leaf volatiles was responsible for behavioral changes in the plant. The results supported the hypothesis, where the blend containing cues that are also shared with the plant was responsible for eliciting the greatest response in *S. tuberosum* and *L. decemlineata*. More broadly, this work emphasized how certain sensory cues in natural and managed systems are pervasive and used by multiple interacting trophic levels. I discuss the potential of applying shared cues, as opposed to single target organism-based treatments, to achieve a magnified effect beyond a single target trophic level.

Dedicated to

Margaret and Joni for their unyielding love and support

# BIOGRAPHICAL SKETCH

Nicholas was raised in the high desert of Arizona where the immensity of the landscape and the biota inspired a deep interest in ecology. His childhood was spent in the canyons, creeks, and caves, of the remarkably diverse environment that is the Southwestern United States. When he was not exploring you could find him in his parent's garage or any number of forts building and experimenting with mechanical and digital creations. This love for innovation and problem solving has followed Nicholas throughout all of his endeavors in life and had a strong influence on his dissertation work and career goals. His passion for innovation can be distilled to a singular guiding principle; to find original and creative solutions to the problems that we increasingly face.

Before coming to Cornell, Nicholas studied at Northern Arizona University at the School of Forestry where he received a BS and MS. During this formative time, he worked closely with Dr. Richard Hofstetter to help test novel treatments for forest insects, with a particular focus on bark beetles. While the lab took part in various semiochemical trials, Nicholas's thesis research was largely focused on developing an acoustic treatment to reduce bark beetle induced tree mortality. Through the combination of working with olfactory and acoustic modalities he became fascinated by the idea of developing insect treatments that target the sensory systems of the plants and insects we aim to manage.

# ACKNOWLEDGEMENTS

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My advisor Jennifer Thaler bears a great deal of responsibility in my development as a scientist. Through the combination of intellect, kindness, and passion for research she mentors her students in a way that allows their true strengths and interests to shine through. Jennifer was always willing to listen to my ideas, no matter how farfetched, and helped me find ways to incorporate them into my own research program. My committee members, Greg Loeb and Andre Kessler, provided the perfect balance of my interests in both applied and basic research and contributed greatly to my professional growth. Several other professors and scientists in the department and related departments also played an important role during my dissertation work. I am deeply appreciative to have developed relationships with Katja Poveda, Todd Ugine, Will Wetzel, Ivan Keresztes, Nicolas Jean-Baptiste, Clare Casteel, Anurag Agrawal, Charlie Linn, Tony Shelton, Rob Raguso, Elson Shields, Art Agnello, Kyle Wickings, and Georg Jander. My fellow graduate students also played an invaluable part during my time at Cornell, none more than Zoe Getman-Pickering and Julie Davis. I am sincerely grateful to have them as friends and colleagues. Additionally, I would like to thank the many undergrads that were in our lab during my time at Cornell who helped make the research in this dissertation possible, including George Stack, Angeliki Cintron, Tait Stevenson, Emma Weissburg, Mayra Miranda, Ari Grele, Abby Dittmar, and Fiona MacNeill. This is especially true for Ari, Abby, and Fiona, who I was fortunate enough to serve as a mentor for their thesis projects. I would also like to thank the staff and administrators for their help along the way. There are too many to list, but in particular Stephanie Westmiller, Darin Gillenwater, Lisa Marsh, and Rita Stucky have always been extremely helpful. Being able to conduct field research has been critically important to my dissertation work, and Steve MacKay at the Thompson Research Farm was always happy to work with my project plans that involve a virulent agricultural pest.

Finally, I would like to thank the funding sources that allowed me to carry out my research. I am profoundly grateful for the award I received from the National Science Foundation Graduate Research Fellowship Program (GRFP), which gave me the opportunity to focus deeply on my research. I am also grateful for the Extension and

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# CHAPTER 1

## Ontogenetic changes and semiochemical release in a primary scent gland of *Podisus maculiventris*

Nicholas C. Aflitto, Abigail Ditmar, Todd Ugine, and Jennifer S. Thaler

### Abstract

*Podisus maculiventris* (spined soldier bug) is a generalist Pentatomid predator studied for its biocontrol potential. This species maintains a series of exocrine glands, the largest of which is the sexually dimorphic dorsal abdominal gland, which is much more pronounced in males. Releases from this gland are used as a pheromone to recruit conspecifics, and an important semiochemical for other organisms within perceptual range. Male dorsal abdominal gland semiochemicals change the behavior of the widespread crop pest *Leptinotarsa decemlineata* (Colorado potato beetle) and they manipulate potato plant physiology. Despite our growing understanding of the species interactions elicited by the compounds in this gland, its development and the conditions that cause the release of compounds remain largely unknown. To better understand gland development and pheromone release behavior, we dissected male bugs at three adult stages and quantified the chemical composition and amount of each compound contained in the dorsal abdominal gland. We also counted the number of sperm present in the seminal vesicles of the three ages of males to determine if gland development was related to sexual maturity. Finally, we measured the diurnal release patterns of different aged males and in various male-female combinations. We found that newly eclosed adults have under-developed glands and no sperm. One week post-eclosion the glands contained the previously reported compounds and viable sperm. Mirroring this trend in reproductive maturation, the number of pheromone releases increased with age and the majority of releases followed a scotophase pattern unaffected by the sexual composition of the predator group. These findings add new information about the development of exocrine glands and release behavior in Pentatomidae and improve our understanding of how these olfactory cues modulate species interactions.

## Introduction

Insect predators play a critical role in food web dynamics and pest management (De Clercq, Peeters, Vergauwe, & Thas, 2003; Polis & Strong, 1996; Tipping, Holko, Abdul-Baki, & Aldrich, 1999). Often, the focus of a predators' role in trophic interactions is on the consumption of prey or the consumptive effect predators have on the prey population. Another way predators interact with prey is by eliciting phenotypic responses in prey that increase their chances of survival, or non-consumptive effects (Lima & Dill, 1990; Sih, 1980; Werner & Peacor, 2003). Because non-consumptive effects are mediated exclusively via sensory cues (Weissburg *et al.*, 2014), understanding the development and release behavior of predator cues will improve predictions about the effects on prey. A heavily relied-upon sensory modality used by insect predators and prey is olfaction. When considering Pentatomids, aptly named stink bugs, the use of chemical communication may be especially pronounced. In addition to prey, chemical cues are also an important source of sensory information for other trophic levels, and the volatile chemicals released from stink bugs have the potential to affect the behavior of non-prey organisms. In a related vein, research has shown that cues from below-ground natural enemies and herbivores can alter plant physiology and make plants more resistant to herbivore feeding (Helms, De Moraes, Mescher, & Tooker, 2014; Helms, De Moraes, Tooker, & Mescher, 2013; Helms et al., 2019). Because stink bugs release many volatile compounds, often in close proximity to or even while standing on plants, the potential for cue interception by plants and other organisms beyond their prey is likely.

The spined soldier bug (*Podisus maculiventris*), is a common omnivorous Pentatomid stink bug distributed across North America (Legaspi & Legaspi, 2007). It is a voracious consumer of numerous insect species, including agricultural pests, and has been released throughout its native range for augmentative biological control and in Europe for inoculative biological control (Biever, K D Chauvin, 1992; Hough-Goldstein & McPherson, 1996). A wealth of research has been conducted on this stink bug species to determine how to use it effectively for biological control (Tipping et al., 1999), with a particular focus on its chemical ecology. This species produces numerous volatile chemicals that are released via a series of exocrine glands (Aflitto & Thaler, 2020; Aldrich, 1988; Hermann & Thaler, 2014; Sant'Ana, Bruni, Abdul-Baki, & Aldrich, 1997). Of specific interest is the dorsal abdominal gland (DAG) from which males can release >1mg of pheromone into their surrounding environment (Aldrich, 1995). This strong olfactory cue has long intrigued chemical ecologist raising questions regarding its importance as an inter- and intraspecific cue. Note that for the purposes of this paper, we refer to the odors released from the DAG that are used for intraspecies communication as a *pheromone*, however when referring to the perception of the pheromone by other organisms we refer to it as a *semiochemical*. Recent work has highlighted the importance of interspecific detection of the DAG semiochemical. Colorado potato beetles (*Leptinotarsa decemlineata* (Say), an economically important crop pest of solanaceous plants, reduces plant consumption in the presence of DAG semiochemicals (Aflitto & Thaler, 2020, 2021; Hermann & Thaler, 2014). Adding to the intrigue involving these semiochemicals is the discovery that plants are also able to detect and respond to the DAG compounds in ways that benefit the plants. Work by Aflitto et al. has revealed there are shared chemical compounds between the DAG and plant green leaf volatile signaling compounds that, when received by the plant, make leaf tissue more defended against herbivorous prey. These plant changes elicited by the stink bug semiochemical cause *L. decemlineata* to feed less on potato plants and hasten flowering time (Aflitto & Thaler, 2021).

Here, we investigate three primary factors that may influence the presence of the DAG semiochemical in the environment: 1) male adult ontogeny and sexual maturation and the development of the DAG, 2) diurnal release patterns, and 3) interactions with conspecifics. Release of the pheromone from the DAG is controlled by opening and closing ostiole located on the abdominal dorsum of bugs. However, the factors that elicit releases from the gland are not well described. More evidence linking the DAG development with other physiological processes such as sexual maturation may uncover a more nuanced use of this semiochemical cue and contribute to a greater understanding of the ecological effects of this source of chemical information in a system. Ontogentic changes in *P. maculiventris* gland development from the immature to adult stage has been well studied (Aldrich *et al.*, 1984b). However, less is known about changes in gland volume and composition that occur during the adult stage in *P. maculiventris,* and more broadly Pentatomids (see Mcbrien *et al.*, 2001; Cribb *et al.*, 2006). To better understand how adult age affects gland development and emissions, we investigated how the amount and composition of DAG chemicals change from the point of adult male eclosion to maturity at 14 days old. To learn more about the potential congruencies between gland development and sexual maturity, we also removed seminal vesicles from males to assess the level of maturity at the same age stages as the DAG measurements. Finally, to understand the behavioral and diurnal conditions that elicit the release of the DAG, we used the same age cohorts mentioned above and male and female combinations to measure the total number of volatile chemical releases through the day and night.

## Methods

### *Podisus maculiventris* Dorsal Abdominal Gland Biology

The contents of the male DAG have been extensively characterized by Aldrich and collaborators (Aldrich, Blum, Lloyd, & Fales, 1978; Aldrich, 1995; Aldrich, 1985, 1988; Sant'Ana et al., 1997). Both male and female bugs have DAGs, however it is many times smaller in females (Aldrich et al., 1978) and there is only a single compound in the female gland in common with those found in males (Aldrich, Kochansky, Lusby, & Sexton, 1984). In male bugs the DAG is the largest exocrine gland, the largest organ in their body, and the site where this species manufactures most of its pheromones. Previous reports demonstrate that the stink bug pheromone aggregate nymphs and adults, especially as overwintering bugs emerge in the spring when the compounds play a role in mate attraction (Aldrich & Cantelo, 1999). The nymphal stages are also attracted to a synthetic blend of the 3 most abundant compounds in the DAG, (*E*)-2-hexenal, α-terpineol, and benzyl alcohol (Sant'Ana et al., 1997). While all motile stages are attracted to the DAG pheromone, the benefit to the individual releasing the volatile cue is not completely resolved. The attraction of both males and females to aggregation pheromones has been reported in other Pentatomid species (Aldrich et al., 1991; Harris & Todd, 1980; James, Mori, Aldrich, & Oliver, 1994). Some of the volatile chemical components of the male DAG pheromone were sold commercially to recruit wild *P. maculiventris* into crops to help suppress pests (Aldrich, Kochansky, & Abrams, 1984).

### *Insects*

*P. maculiventris* were maintained in a laboratory colony and were provided an *ad libitum* diet of *Tenebrio molitor* L. larvae and greenhouse-grown 2-5 wk-old *Solanum tuberosum* L. plants (cv. Yukon Gold). The colony was maintained at 25 ± 2º C, ambient relative humidity and a 16:8h L:D cycle.

### *Male gland development over time*

To better understand the relationship between male DAG maturity and sexual development we characterized the size and chemical composition of the contents of the male DAG at three time points. To do this, we generated discrete cohorts of fifth-instar nymphs weekly for the three weeks leading up to the start of the experiment. This allowed us to have adults that were one day post-eclosion (young), seven days post-eclosion (mid), and fourteen days post-eclosion (old). Each cohort was kept in a separate cage and once eclosed, all females were removed from the cups to ensure no males mated with females.

Individual males were removed from their respective colony (age class) and anesthetized with $CO_2$. Insects were then weighed to the nearest mg and we measured and recorded their length and width at their widest point using digital calipers. Specimens were then dissected under PBS with the aid of a microscope. We removed the DAG (Fig. 1) and measured its length and width at the widest points before quickly transferring it to a 2 ml auto sampler vial containing 200 µL of dichloromethane. We measured five glands from each of the 3 weekly cohorts, for a total of 15 replicates. All gland samples were stored at -80º C until GC-MS analysis.

One hundred microliters of each DAG extract were transferred to 150 µL inserts contained within autosampler vials. We added 10 µL of tetralin, (90 ng/mL) to each sample as an internal standard. Because the male DAG contains many compounds at a wide range of concentrations, we ran each sample using a split setup at 3 sensitivities. This allowed us to more accurately quantify the focal analytes at the variable amounts present in the age cohorts. The peak area of each compound was calculated and made relative to the internal standard peak to obtain the concentration of the detected compounds.

In addition to the gland measurements, we removed one seminal vesicle from each individual, and carefully placed them in 2 mL centrifuge tubes containing 1 mL of PBS solution. We gently ruptured the vesicle with fine forceps to release sperm. We made three serial dilutions (1:5, 1:10, and 1:20) of the original 1 mL stock to ensure that we had a concentration of sperm that could be easily quantified. Ten microliters of each dilution were then pipetted on 15-well Multitest slides (MP Biomedicals, LLC) with predefined 3 mm diameter circles. We counted the number of sperm from 2 wells for 10 individuals from each age cohort and used the average of the two estimates for statistical analyses after accounting for dilutions.

### *Pheromone release frequency*

We conducted a series of experiments to try to better understand the factors the elicit male DAG release behavior. To determine the effect of male age on DAG release frequency, we paired two 'young' males (<1 day post eclosion), or two 'old' males (7-14 days post eclosion). Pairs of young or old males were placed into 237 ml glass jars, which were part of our olfactometer described in full in Aflitto and Thaler (2020). Briefly, the olfactometer was set to a constant airflow rate of 50 ml of charcoal-filtered air per minute. The filtered air entered each jar, was passed over the pair of bugs before it was exhausted from the jar and passed over a metal-oxide volatile organic compounds (VOC) detector (IONTIK SemioSensor). These sensors log total VOCs, time, humidity, and temperature to a microSD card at a rate of 1 sample per second, an equivalent of

86,400 data writes per 24 h replicate. We performed the experiment on four different days with new pairs of males of each age cohort on each day.

Next, we performed an experiment to determine whether the presence of females influenced release behavior. To do this, we compared the number of releases of pairs of same-aged males (7-14 d, n=10 replicates), pairs of females (age-range: 6 reps) and mixed-sex pairs (1 male with 1 female, n=10 replicates). Treatments were randomly assigned to days and each replicate was conducted on a different day. Data was collected for 24 h for each replicate as described above. For all sensor data, blank runs containing no bugs were conducted as a negative control to confirm the detection of bug-related releases. Preliminary testing indicated that a DAG release elicited sensor deflections ≥ 0.2 percent from the baseline reading. This helped filter out other VOC sources coming from the bugs such as defecation and smaller exocrine gland releases.

### *Statistical analysis*

Significance was tested using linear models. Linear mixed effects models were used for gland and body size comparisons and release behavior with age as a fixed effect and date of measurement as a random effect. Linear models were used to assess chemical compound differences and for the seminal vesicle comparison with age as a fixed effect. Linear mixed effects models were used for pheromone release behavior comparisons, where treatment and time of day were included as fixed effects and date as a random effect. Post hoc analyses were completed using estimated marginal means. All analyses were completed using R statistical software (R Core Team 2014). Data was transformed where necessary to meet the linear model assumptions of normality and homogeneous variance. Mixed models were analyzed using the lme4 package (Bates, Maechler, Bolker, & Walker, 2015).

## Results

### *Dorsal Abdominal Gland and Sexual Development*

While the mass of each age class of adults was similar ($F_{2,99} = 0.011$, p = 0.9), the body size (length x width) differed significantly ($F_{2,102} = 13.57$, p = <0.001). Fourteen day-old adults were slightly smaller (5% smaller) than the younger age groups. The size of the DAG differed as a function of the time post-eclosion ($F_{2,47} = 41.61$, p = <0.001). We observed that 1 day-old bugs had substantially smaller DAGs compared to the 7 and 14 d-old males, which did not differ from each other (Figs. 1.1 & 1.2a).

We found that seminal vesicles in 1 d-old adult males did not contain sperm. Sperm count increased with time post-eclosion, with 7 d-old males containing 492 sperm per vesicle (984 total) and the 14 day-old averaging 1087 per vesicle (2174 total). Total seminal vesicle sperm count increased with age and was different for each age cohort (1d vs. 7d: T = -7.72, df = 20, P = < 0.0001) (7d vs. 14d: T = -3.62, df = 20, P = 0.005) (1d vs. 14d: T = -10.67, df = 20, P = <0.0001) (Fig. 1.2b).

### *GC-MS analysis*

Several compounds were found in the mature DAG, however we were able to confirm 5 dominant compounds; (*E*)-2-hexenal, alpha terpineol, benzyl alcohol, terpin-en-4-ol and linalool (Fig. 1.3). Newly eclosed adult males only contained trace amounts of (*E*)-2-hexenal and some individuals contained tridecane (Fig. 1.1, Table 1.1). Additional terpenoid compounds were also found in the mature glands that remain to be identified (tentatively identified as d-limonene and p-cymene) (Fig. 1.4).

*Pheromone release frequency*

We found a strong effect of male age on the frequency of releases from the DAG (T = 6.57, df = 4, p = 0.003). We did not detect any releases from pairs of newly eclosed males. In contrast to the lack of releases in newly eclosed males, we detected an average of 3.95 releases per hour from pairs of 7-14 day old males over the 24 hour period (Fig. 1.5). There was a strong diurnal pattern in releases from pairs of older males (T = -7.31, df = 89, p < 0.001). We observed 8.79 releases per hour during scotophase (Fig.1.6).

We detected significantly fewer releases from pairs of females (1.25 releases per 24 h) compared to male-female pairings (30.6 releases per 24 h) (FF-MF: T = -3.99, df = 17, p = 0.003) (FF-MM: T = -4.53, df = 17, p = 0.0008) (Fig. 1.7). We did not find any evidence that the number of DAG releases was influenced by the presence of females (MM/MF comparison: T = 0.643, df = 14, p = 0.799), and detected similar numbers of DAG releases from pairs of males and mixed male-female pairs.

## Discussion

We found that as male *P. maculiventris* mature, their DAG gland size increased, and the chemical profile of the DAG became more complex with several compounds becoming more abundant. These changes were directly linked to male sexual maturity. The glands of 1-day-old adult males were nearly devoid of the compounds that dominated the profile of the older, more mature males that we analyzed. The chemical composition of the DAGs of 7-d old adults contained all of the previously documented compounds presented in Aldrich (1984b), and at 14 d-post eclosion we found the same suite of compounds, but they were more abundant. The gland development coincides with our proxy for sexual maturation, with the young age cohort containing no viable sperm and 7 and 14-day adults having 492 and 1087, respectively.

The VOC sensors detected the greatest number of VOC emissions from the late evening to early morning (20:00-6:00h). It is important to emphasize that we measured the total number of releases from bugs using metal-oxide sensors that detect total VOCs and did not measure the specific compounds released. It was previously reported that emissions from the DAG occur during the day, with some males remaining volatilely silent on some days (Aldrich et al., 1984). The results from our temporal sensor measurements found what looks to be a scotophase pattern in releases, and that newly eclosed males, with their underdeveloped glands, might be what has been described as silent individuals. The delayed release of semiochemicals in non-sexually mature males is similar to what has been shown in *Halyomorpha halys* where adults begin emitting pheromone after ~13 days (C. Harris, Abubeker, Yu, Leskey, & Zhang, 2015). Another

Pentatomid, *Piezodorus hybneri* is also known to not release pheromones until sexually mature (Endo et al., 2007). Further, knowing that immature males do not release the DAG pheromone, and that in mature males it is primarily released in scotophase may explain variability in previous trials using live male *P. maculiventris* as an odor treatment source. Additional work is needed to confirm which specific compounds are being released in the evening and that they come from the DAG. Scotophase releases of volatiles and attraction to pheromones has been shown in other Pentatomids (Krupke, Jones, & Brunner, 2006). For example, *Halyomorpha halys* releases a defense compound in greater amounts in the evening and night (C. Harris et al., 2015). Our data shows the greatest amount of VOC releases occurring during the evening to early nighttime hours. Interestingly, knowing that diurnal prey detect and respond to DAG semiochemicals, a scotophase release pattern may help *P. maculiventris* evade detection by its prey. The nighttime release may also have additional benefits such as evading detection of its own predators and parasitoids and by releasing at a time when the air is cooler and more humid.

These findings provide additional insights into the ontogenetic changes and the timing of release regarding the DAG, which is the first described aggregation pheromone in true bugs (Aldrich et al., 1978). In a field trial using a synthetic DAG blend containing three of the most abundant compounds, it was found to attract both males and females and confirmed the use of the DAG contents as a long-range aggregation pheromone (Aldrich et al., 1984). Learning that the development of the DAG pheromone coincides with sexual maturation in males provides support to the hypothesis that the gland is used to attract mates and as an aggregation pheromone. Although both males and females are attracted to the pheromone, males that are attracted may be exploiting the signal in the hopes of finding a mate.

Interestingly, the 1 day-old adults were the only age cohort to contain the alkane tridecane. This compound is found in immature sting bug DAG's and  may be residual material left over from the nymphal stages (Aldrich et al., 1984). Tridecane has been described as an important pheromone for another Pentatomid, *Nezara viridula*, where the nymphal stages release tridecane as an aggregation pheromone to remain clustered (Lockwood & Story, 1985). The amount of tridecane increases as they mature through the 5 nymphal stages and higher volumes of the compound in the air elicits anti-aggregation behavior, causing the later nymphal stages to disperse. Others have found that tridecane, in addition to (*E*)-2-hexenal, to be an effective repellent of other arthropods. The Pentatomid *Coridius janus*, releases a defense secretion containing the bioactive components of tridecane and (*E*)-2-hexenal which repelled 3 herbivorous insects (Gunawardena & Herath, 1991). The presence of tridecane in the newly eclosed males in our study might provide a level of protection from other insects while they are callow and quite fragile, however more research is needed before this is substantiated.

From an applied perspective, gaining a more complete understanding of the effects of the DAG semiochemical on crop pests will allow growers to better leverage the volatile cue for plant protection against herbivores. Hermann and Thaler (2014) showed that the presence of adult male *P. maculiventris* contained in breathable bags changed *L. decemlineata* behavior; beetles exposed to stink bug semiochemicals in the field reduced the rate of potato leaf consumption. However, the long-term use of live predators as a semiochemical source can elicit a variable response by *L. decemlineata* over a season and interannually (Aflitto & Thaler, 2020). The use of a synthetic DAG mixture in release devices, which provide a more consistent release of the semiochemical, has been shown to produce a more reliable response, causing *L. decemlineata* to avoid plots containing DAG emitters and reduce plant damage (Aflitto & Thaler, 2020, 2021). In 2020, a synthetic blend of the male DAG was approved as an organic crop treatment to reduce *L. decemlineata* plant damage (Aflitto and Thaler 2020). This difference between the response of potato beetles to live stink bugs and synthetic pheromone emitters raises questions about the consistency of cue release from the live stink bugs and the conditions that elicit their release. It also raises questions about development of the DAG and behavioral changes affecting the release of the gland contents to maximize plant protection.

Male *P. maculiventris* have the ability to release large amounts of volatile chemicals from their dorsal abdominal gland into the environment, making it a strong cue source for their prey and other trophic levels to perceive and interpret. We found the presence of this sensory source to be closely linked to the male sexual development and temporal patterns. We also found a profound effect of male age on the chemical profile of the DAG. These findings will aid in understanding the ecological interactions that are mediated by this strong semiochemical, such as interactions with their prey and plants.

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Figure 1.1 Adult *Podisus maculiventris* dorsal abdominal gland (DAG) dissection comparison across the 3 age cohorts of males. Yellow arrows point to the DAG.

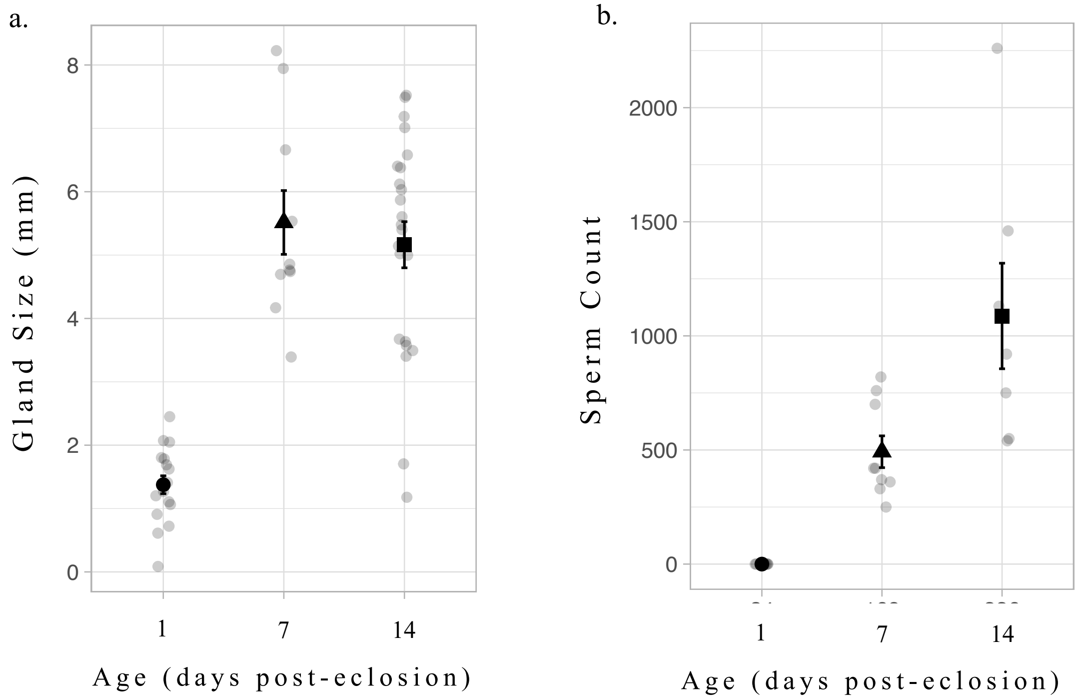

Figure 1.2 a. The mean gland size (length x width) of 1-, 7-, and 14-day old male *P. maculiventris* adults. b. Sperm count from the seminal vesicles of each age cohort. Age is represented by days since eclosure. Bars represent standard error of the mean.

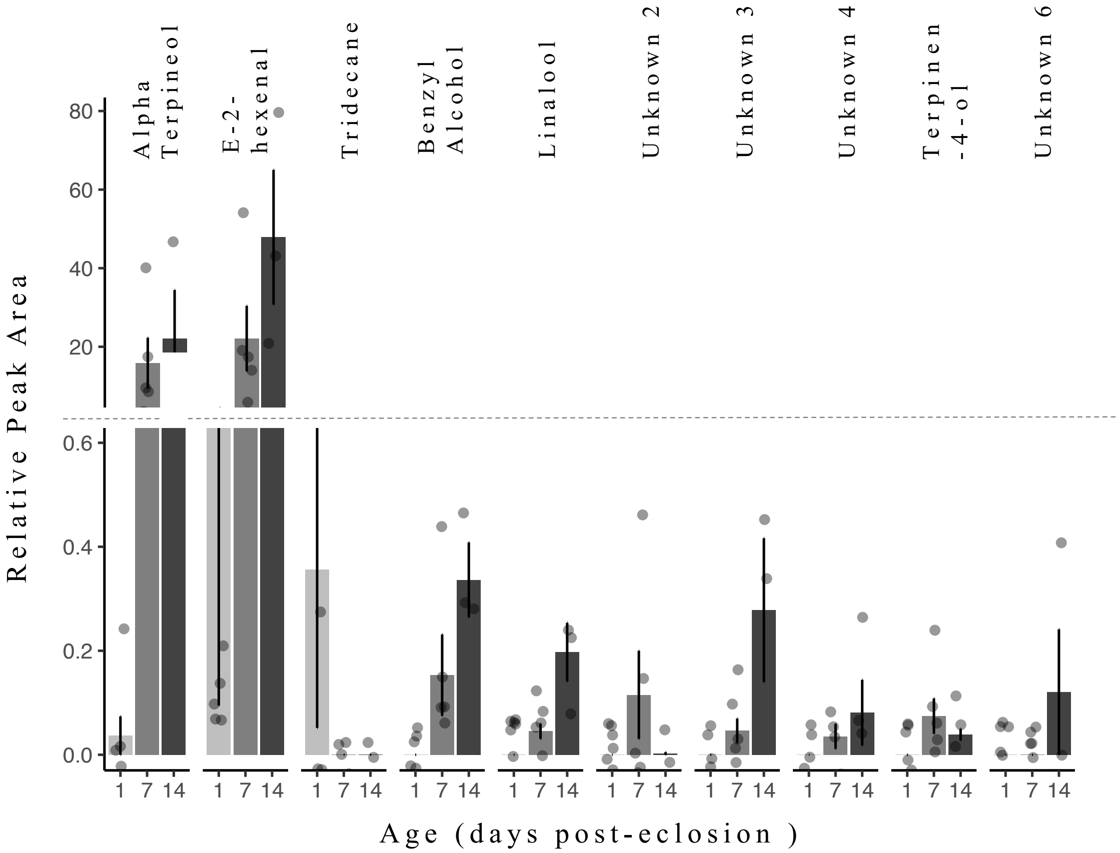

Figure 1.3 Mean (±SE) peak area for each compound expressed relative to the internal standard (tetralin) for 1-, 7-, 14-day post-eclosion *P. maculiventris* males. Note that (*E*)-2-hexenal in the 1-day cohort has a relative peak area of 2.3 and is not shown for figure readability purposes. [unknown compounds awaiting NIST library access].

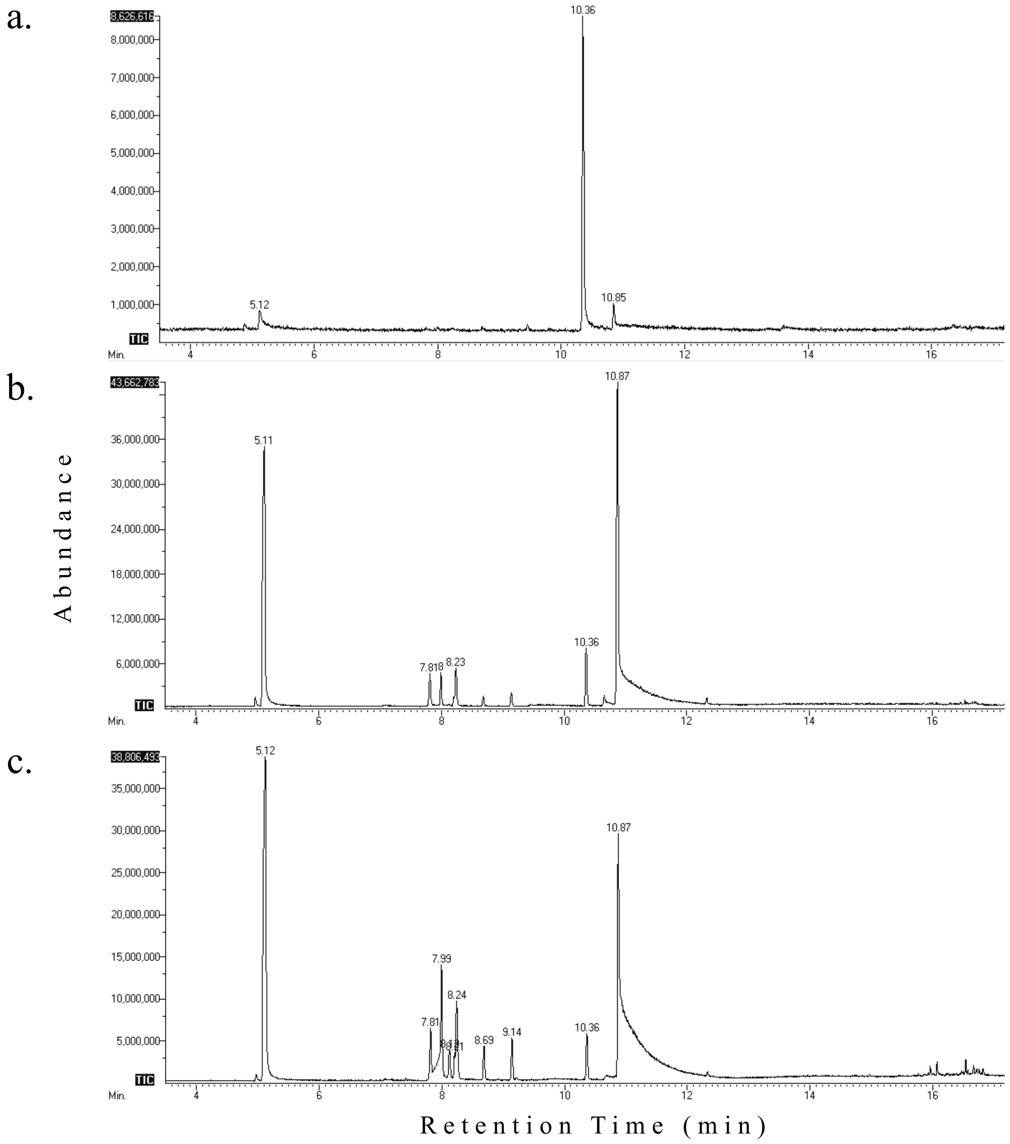

Figure 1.4 Representative gas chromatograms of the content of the dorsal abdominal glands for each age cohort of *P. maculiventris* males. Panel (a.) is representative of 1-day old age cohort, (b.) 7 day old, and (c.) 14 day old. The 10.36 peak is the internal standard, 5.12 is e-2-hexenal and 10.87 in alpha terpineol. Note the y axis scale differences.

Table 1.1 Mean amount (µmol) per *P. maculiventris* male and standard error of each compound identified in the GC-MS analysis.

| Compound<br>(Retention Time in mins) | Young<br>(µmol±**SE**) | Medium<br>(µmol±**SE**) | Old<br>(µmol±**SE**) |
|---|---|---|---|
| **(*E*)-2-hexenal (5.09)** | 2.3±2.6 | 22.1±8.3 | 50.2±17.1 |
| **benzyl alcohol (7.8)** | 0±0 | 0.2±0.08 | 0.34±0.07 |
| **7.99** | 0±0 | 0.1±0.08 | 0.003±0.003 |
| **linalool (8.12)** | 0±0 | 0±0 | 0.1±0.1 |
| **8.20** | 0±0 | 0.1±0.03 | 0.3±0.1 |
| **8.69** | 0±0 | 0.04±0.02 | 0.08±0.06 |
| **terpinen-4-ol (9.14)** | 0±0 | 0.07±0.03 | 0.04±0.01 |
| **alpha terpineol (10.89)** | 0.04±0.05 | 15.8±6.5 | 22.1±12.4 |
| **tridecane (12.32)** | 0.4±0.3 | 0±0 | 0±0 |

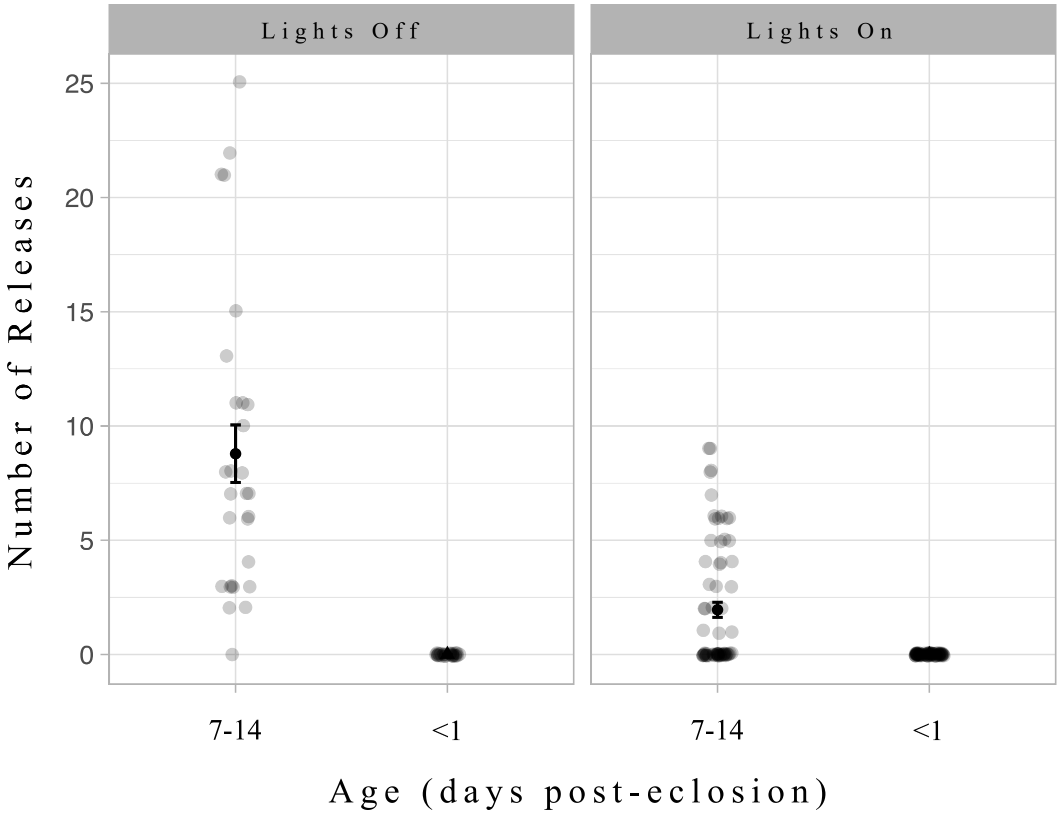

Figure 1.5 Mean number of releases per hour for the old and young *P. maculiventris* male cohorts with standard error bars. Each dot represents the mean total number of sensor deflections (>0.02%) for a replicate male bug. The left panel is data collected when the lights were off, and the right is when the lights were on. Bars represent standard error of the mean.

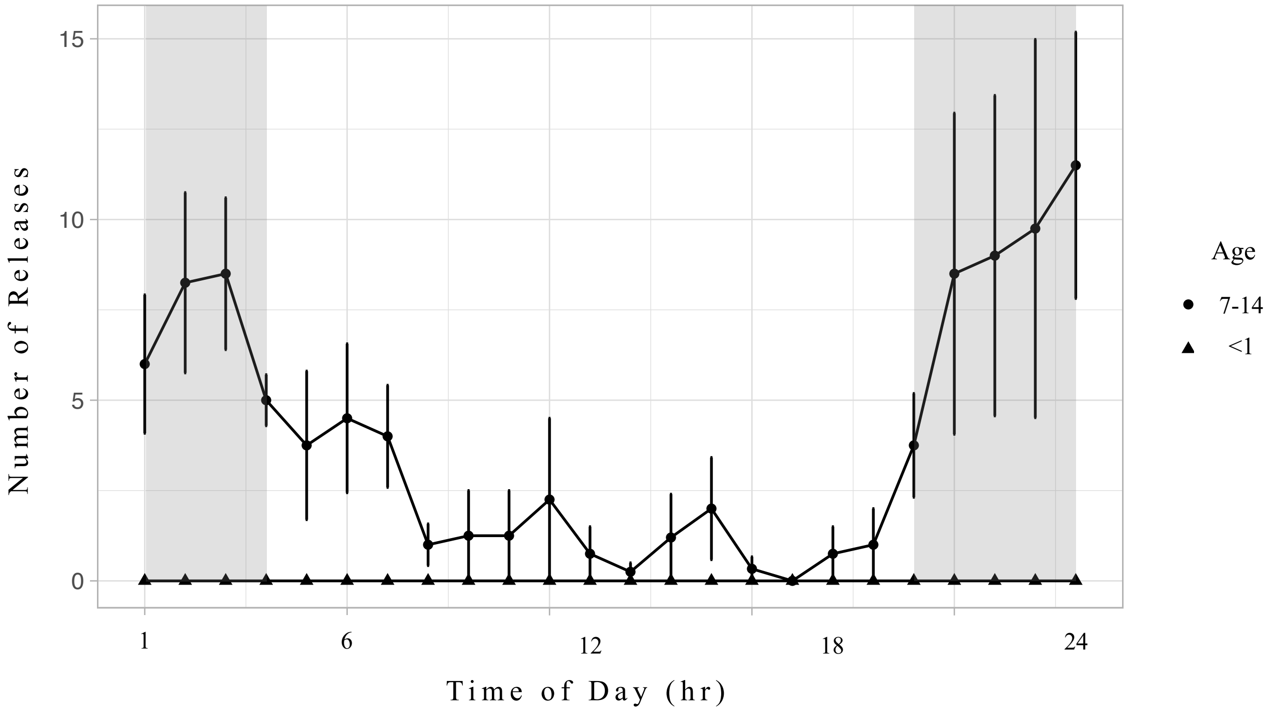

Figure 1.6 Mean number of releases per hour for newly eclosed (<1 day) and mature cohorts (7-14 day) *P. maculiventris* males with standard error bars. Lights in the arena were turned on at 4:00 and turned off at 20:00 UTC (shaded grey zone). Bars represent standard error of the mean.

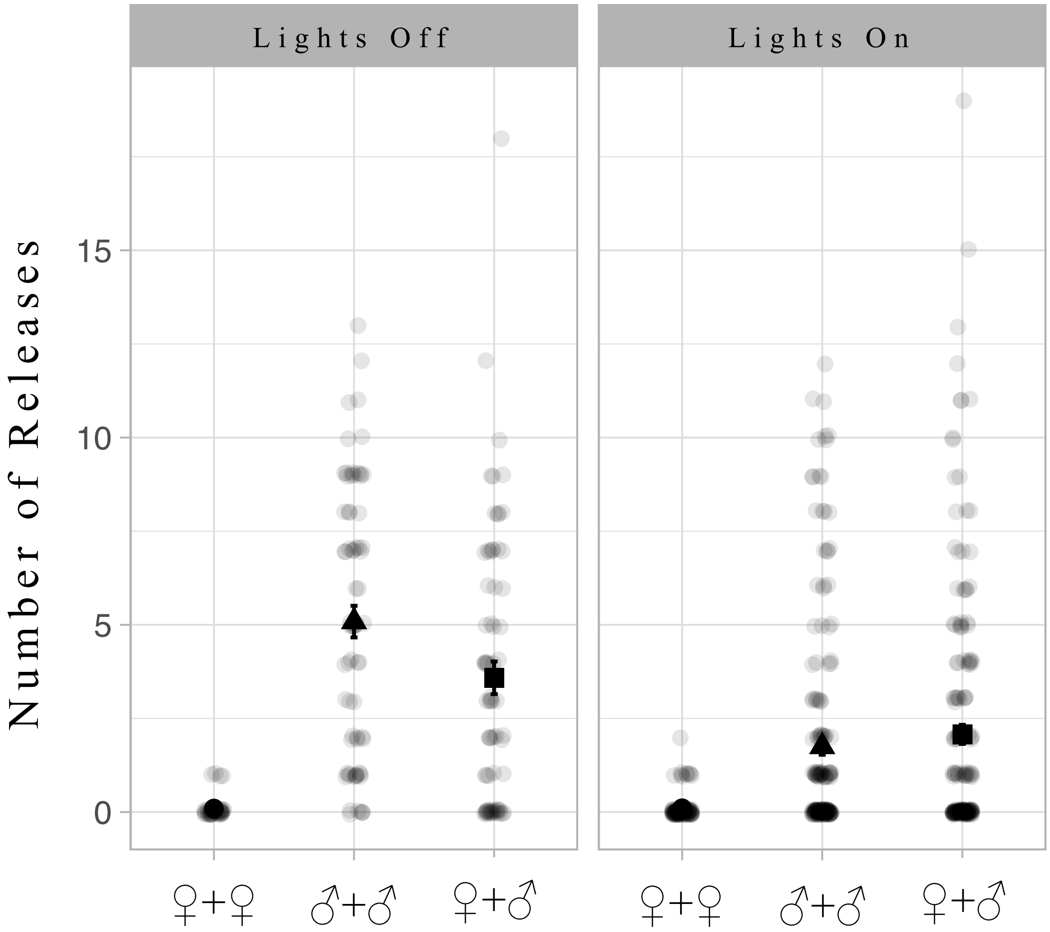

Figure 1.7 Mean number of *P. maculiventris* releases per hour of two females, two males, and a male with a female with standard error bars. The right panel is when the lights are off, and the left is the lights on. Bars represent standard error of the mean.

# CHAPTER 2

# Predator pheromone elicits a temporally dependent non-consumptive effect in prey*

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## Abstract

The influence of predator cues on the behavior of prey is well supported in the literature; however, a clear understanding of how predator cues affect prey in variable environmental conditions and over longer time scales is needed to better understand the underlying mechanisms. Here, we measure how predator odors affect herbivore colonization, abundance, oviposition, and plant damage across two growing seasons. The study system consisted of *Leptinotarsa decemlineata* (Colorado potato beetle) as prey, and the aggregation pheromone of live *Podisus maculiventris* (spined soldier bug) as the predator cue in a potato field. In 2016, the amount of feeding damage by early beetle colonists was lower in predator odor-treated plots, reducing plant damage by 22%. Larval abundance was also reduced in treated plots in 2016. Beetle abundance and damage in 2017 was similar in the treatment and control plots. Two mechanisms were investigated to better understand why prey response to the predator odor treatment weakened over the first season, including changes in predator odor cue strength and prey habituation. Predator odor cue strength emerged as a likely explanation, as dispensers, which released a synthetic predator pheromone over the entire season, reduced the probability of finding damage more consistently than the live predator treatment. These results suggest that temporal patterns of predator cue release and strength may drive prey response across the season, underscoring the importance of cue release-rate and consistency in both species interactions and for the future application of modifying insect behavior using non-consumptive effects in agricultural systems.

Key words

applied chemical ecology, behavior modifying compounds, biological control, non-consumptive effects, trait-mediated indirect interactions, predator–prey interactions, sensory ecology

## Introduction

The risk of predation is a fact of life for nearly all organisms. Predator cues can elicit phenotypic changes in prey (Lima & Dill, 1990; Sih, 1980; Werner & Peacor, 2003), including behavioral (Morrison, 1999; Nelson, Matthews, & Rosenheim, 2004), morphological (Dixon & Agarwala, 1999; Weisser, Braendle, & Minoretti, 1999), and/or physiological (McPeek, Grace, & Richardson, 2001; Slos & Stoks, 2008), jointly termed non-consumptive effects (NCEs). Although the existence of NCEs are well documented, a clearer understanding of how predator cues affect prey over longer time scales is needed to reveal more about the effects on prey behavior and population dynamics (Creel, 2011). From an agroecology perspective, little is known about how these interactions function in field settings over seasonal and interannual time scales, reflecting the progression of ontogenetic stages in crop pests, predators and plants.

While the majority of NCE studies are completed on short time scales (Hermann & Landis, 2017; Preisser, Bolnick, & Grabowski, 2009), many mechanisms operating over longer time scales have the potential to alter the strength of NCEs. The predation risk allocation hypothesis states that prey must balance foraging needs and anti-predation measures depending on the temporal changes in perceived risk (Lima & Bednekoff, 1999). When a short study length is used, and a pulse of predator cues are presented to prey, mechanisms such as non-associative learning (i.e. habituation) (de Vos, Cheng, Summers, Raguso, & Jander, 2010; Roberts, 2014) might not be uncovered (Hoverman & Relyea, 2012). Prey responses can also change depending on their development stage (Thaler & Griffin, 2008) or prey morphology (i.e. larger prey are less vulnerable to predation and may be less sensitive to predator cues) (DeWitt, Sih, & Hucko, 1999). Furthermore, it is important to know how the effects of predator cues change with prey development stage, as prey life stage alters how they perceive their environment. For example, adult insects may have more developed vision (Sherk, 1978) or olfactory organs (Dickens, 2002) than larvae, altering prey's ability to detect predator signals. Similarly, the range of sensory cues emanating from predators can vastly change depending on the gender (Hermann & Thaler, 2014), development stage (Gupta, 1961; Moody, 1930), and physiological condition (Lefcort, Ben-Ami, & Heller, 2006; Scherer & Smee, 2016). For example, insect predators can completely change their physical appearance from larva and nymph to adult. Olfactory cues from these different life stages can also vary with the development of exocrine glands as sexual maturation and other processes allow.

Finally, environmental heterogeneity can also alter predator cue propagation, and the ability of prey species to reliably detect such sensory cues (Weissburg, Smee, & Ferner, 2014). For example, insects are highly dependent on olfaction (Dethier, 1947; Hansson & Stensmyr, 2011) and chemical cues can be greatly affected by air turbulence (Elkinton & Carde, 1984), temperature (Elkinton & Carde, 1984; Linn, Campbell, & Roelofs, 1988), distance and time (Murlis, Willis, & Cardé, 2000) and humidity (Royer & McNeil, 1993; Wiener & Capinera, 1979). By conducting experiments on longer time scales, and by capturing ambient environmental factors such as in field studies, the ability of prey to receive and interpret predator chemical cues in natural environments will be better understood. Studies in agricultural settings will provide growers and land managers with additional knowledge for the future application of predatory semiochemicals to alter insect behavior.

Here we assess the temporal pattern of olfactory-induced NCEs in an agricultural predator-prey interaction. *Leptinotarsa decemlineata* (Colorado potato beetle) was used as the focal prey species due its status as the most important insect herbivore of potatoes (Alyokhin, 2009) and prior studies showing a reduction in plant consumption (Hermann & Thaler, 2014; Thaler, McArt, & Kaplan, 2012) to the odors of the predatory stink bug *Podisus maculiventris* (spined soldier bug). *P. maculiventris* co-occurs with L. decemlineata in agricultural fields and is marketed as a generalist predator biocontrol agent (J. A. Aldrich & Cantelo, 1999; De Clercq, Peeters, Vergauwe, & Thas, 2003). Specifically, we asked three questions: 1) Do predator odors elicit NCEs, expressed as a reduction in prey plant consumption and avoidance of predator odor plots, in an agricultural setting? 2) Do they fluctuate over the growing season and interannually? and 3) what are the potential mechanisms that underlie temporal patterns of NCEs in field conditions?

## Materials and Methods

### Field Study: Temporal pattern of NCEs

On May 18, 2016 and May 19, 2017, *Solanum tuberosum* (cv Yukon Gold) seed potatoes were planted at the Cornell University's Homer C. Thompson Vegetable Research Farm in Freeville, NY (42.519701, -76.334162) using standard potato cultural practices. Both years of the study were planted at the same site. The site was fertilized with triple 13 NPK at a rate of 733kg/ha at the time of planting.

Prior to the emergence of potatoes, experimental *L. decemlineata* were collected from wild populations in the surrounding farm by use of potted potato trap plants. Overwintering adults were collected from trap plants (cv Yukon Gold, 42.519142, -76.334846) every 2-3 days and brought back to the lab until the study start. While waiting to be rereleased, beetles were maintained on Yukon Gold potato plants under a 18:6 (L:D) light cycle. The *P. maculiventris* used in the study were reared and maintained in a laboratory colony, originally collected in Tompkins County, NY. The colony included potato plants and *Tenebrio molitor* larvae as a food source and were maintained under an 18:6 (L:D) light cycle.

### Live Predator Odor Treatments

In 2016 and 2017, fourteen rows of potatoes were planted spaced 0.86m apart, and each plant within a row was spaced by 0.3m. Once plants reached approximately 20cm in height, sixty-six plots (33 control, 33 treatment) in 2016 and forty-one plots (20 control, 21 treatment) in 2017 were established. At this stage, the potato plants were still in early vegetative growth phase, but large enough to attach the treatments without causing damage. Plots were 3 rows wide and 7 plants long. At least 1 buffer row of plants was planted in between all plots, and 1 buffer plant within rows to prevent treatment compounds from affecting adjacent plots, however some drift of compounds moved by wind was possible. Control and predation risk treatments were randomly assigned and installed on the center plant of each plot and consisted of an Agrifabric cylinder (Agrifabrics Pro 17, Alpharetta, GA) that was placed over a portion of the potato plant and secured by flagging on the top and bottom, creating a breathable cage. Ten P. maculiventris (5 ♂, 5 ♀) were introduced to the treatment cages and any missing or dead bugs were replaced during the site visits. Control plots had a blank Agrifabric cage. Three consecutive focal plants were selected in each plot which were measured throughout the study. Focal plant transects ran either east or west from the center, randomized to prevent bias. Each successive focal plant was 0.3m further away from the center treatment cage. Previous work has shown the treatment effect does not expand much beyond a 1m radius when using a similar treatment. Leptinotarsa decemlineata adults, larvae, and egg clutches were removed from all plants starting from the beginning of potato emergence up to the start of plot measurements. To initiate L. decemlineata colonization each year, the wild collected beetles from the surrounding farm were released 24h after the predator treatments were established. In total, 200 beetles were released on June 24, 2016, and 160 beetles on June 20, 2017 at 10 evenly spaced points around the perimeter of the study site. Fewer beetles were released in 2017 to match the same beetle:plant ratio as in 2016. Beetles were then allowed 3 days to establish before measurements began. Note, since the study was conducted in an open field additional wild beetles likely visited the site.

Four plot-level measurements were taken on each of the three focal plants every site visit: number of adult beetles, number of larvae, number of egg clutches, and the amount of new herbivore damage (herbivory proxy). The new herbivore damage was measured on a focal leaf of a focal plant. The focal leaf was marked by a small piece of flagging and during each site visit it was moved to an undamaged leaf. This prevented double counting damage and avoided instances of missing herbivory where the leaf was fully consumed, thus not measurable. Damage was measured with a $mm^2$ grid printed on clear acetate. In total, 7 site visits were completed between June 27 and July 13 in 2016, and 6 site visits between June 23 and July 10 in 2017, all spaced between 2 to 5 days apart, capturing the majority of the growing season for this early season potato variety including emergence, vegetative growth, flowering and the beginning of tuber formation.

For each year, we tested the hypothesis that the treatment *affected L. decemlineata* behavior using mixed models. Hurdle models were used due to the large number of observed zero values in the response variables. A binomial generalized linear mixed model was used to model the probability of the presence or absence of each response variable (plant damage, adult colonization, oviposition, or larval abundance). In each model, treatment, time of season and transect direction were included as fixed effects and plant nested within plot was included as a random effect. A linear mixed model was used to model the effect of the treatment on the non-zero amount of average plot damage and larval abundance. In each model, treatment, time of season and transect direction were included as fixed effects and plant nested within plot was included as a random effect. Average plot damage was log transformed to meet the linear model assumptions of normality and homogeneous variance. Note, the data presented in the figures are not transformed. Linear models were not fit to the oviposition and adult colonization data due to the low number of non-zero values. All analyses were completed using R statistical software (R Core Team 2014, v1.2). Mixed models were analyzed using the lme4 package (Bates, Maechler, Bolker & Walker, 2015) and all post hoc analyses were completed using estimated marginal means package (Lenth, Singmann & Love, 2018).

Weather data was collected from a station (Rainwise IP-100) located at the Thompson Research Farm (42.518687, -76.334033) except for precipitation measurements which were obtained from the National Oceanic and Atmospheric Administration (NOAA 2018). For the analysis, temperature, relative humidity, wind speed, and wind direction were averaged for the days in between site visits and truncated to 7:00am to 7:00pm, which is the time of day the beetles are predominately active. Our goal was to capture the average weather conditions the plants and animals experienced in between site visits. Weather conditions and their potential interactions with the treatment were tested in the linear mixed models for the aforementioned field studies.

## Mechanisms of temporal pattern in NCEs

### *Mechanism 1: Seasonal Changes in Predator Odor Cue*

The largest source of odors in *P. maculiventris* comes from the male pheromone, which was used to test the seasonal changes in odor cue. Since the live predators can control their release of the pheromone, the synthetic pheromone dispensers were used to test prey response to a consistent predator odor cue. In addition, the more consistent odor source allowed us to learn more about the treatment effects on the overwintering and summer beetle generations. While the dispensers provided a more consistent release, it is important to acknowledge that the different vapor pressures of the compounds and the open dispersion volatile dispensers contributed to some variability of the synthetic pheromone treatment. On May 19, 2017, Yukon Gold seed potatoes were hand planted using the same fertilization treatment previously stated in an adjacent field at the Thompson Research Farm. Forty plots (20 control, 20 treatment) containing 9 plants each (3 rows of 3 plants). Each plant was separated by 0.3m and plots were at minimum 3m apart. On June 19, the treatments were installed on the center plants, which consisted of 2mL clear Eppendorf tubes with 3 0.55mm holes in the top, acting as open dispersion dispensers. In plots receiving the treatment, the dispenser was loaded with 594.1µl of a blend consisting of 354.0µl (E)-2-hexenal, 216.0µl alpha terpineol, 2.4µl linalool, 2.0µl terpinen-4-ol, and 19.7µl benzyl alcohol (release-rate of 0.38mg/hr), which are the primary constituents of the *P. maculiventris* aggregation pheromone (Aldrich et al. 1984a) contained in their dorsal abdominal glands. The release-rate was calculated by averaging the weight difference of 3 independent release devices placed in the field for 75 hours ($rr = \frac{(Weight_{time0} - Weight_{time1})}{time_{total}}$). For reference, a single male *P. maculiventris* can release > 1mg at a time (Aldrich, 1995). Control plots received an empty dispenser. Previous lab tests by the authors illustrate NCEs in *L. decemlineata* using this blend (N. Aflitto unpublished). The treatment and control dispensers were replaced every 2 weeks over the study period. The same measurements were taken on all 9 plants per plot as in the live predator plots stated above.

We tested the hypothesis that the synthetic odor dispensers affect L. decemlineata behavior using mixed models. Hurdle models were used due to the large number of observed zero values in the response variables. A binomial generalized linear mixed model was used to model the probability of the presence or absence of each response variable (plant damage, adult colonization, oviposition, or larval abundance). In each model, treatment and time of season were included as fixed effects and plant nested within plot was included as a random effect. A linear mixed model was used to model the effect of the treatment on average plot damage. In each model, treatment and time of season were included as fixed effects and plot was included as a random effect. Untransformed damage measurements met the linear model assumptions of normality and homogeneous variance. Linear models were not fit to the adult colonization, larva, or oviposition presence data due to the low number of non-zero values. All analyses were completed using R statistical software (R Core Team 2014, v1.2). Mixed models were analyzed using the lme4 package (Bates, Maechler, Bolker, & Walker, 2015) and all post hoc analyses were completed using estimated marginal means package (Lenth, Singmann & Love, 2018).

## Mechanism 2: Prey Habituation

In the lab, we crossed beetles pre-exposed to predator odors and naïve beetles in the treatment and control sides of a no choice olfactometer, with the goal of quantifying any feeding effects caused by the pre-exposure of beetles to predator odors. If habituation to predator odors was present, the beetles pre-exposed for the longest time period should reduce their response to the treatment the greatest. In the lab, *L. decemlineata* were reared from eggs to adults and newly eclosed beetles were collected and placed into separate control and treatment cages. Treatment cages consisted of a 27cm (h) x 20cm (w) cylindrical cage containing 10 P. maculiventris (5 ♂, 5 ♀) restricted in an Agrifabric bag (same as field treatment) with a potato leaf in a water pick to allow the predators to plant feed. A potted Yukon Gold potato plant was placed within the cage as a food source for *L. decemlineata*. Adult beetles were exposed to this live predator odor treatment for three time periods: 5, 24 and 168h. Control colonies were the same except for the absence of live predators. Every beetle tested was kept in a separate cage with a separate cohort of predators to maintain replicate independence.

After pre-exposure periods were met, the beetles were immediately tested for their response to predator odors. Beetles were removed from the predator treatment or no predator colonies and placed in a no-choice olfactometer with either predator odors or filtered air (control) moving at 100 mL/min. The olfactometer, constructed by the authors, carbon-filters bench air and splits it between an odor treatment side or a control side. After 24 hours, weight gain and leaf consumption were measured (n=79).

A linear mixed effect model was used to test the hypothesis that beetles are habituating to the predator odors. Relative leaf consumption (relative to starting beetle weight) was used as the response variable and treatment, predator odor exposure and exposure time as fixed effects and date of olfactometer test as the random effect. The effect size between treatments was measured using Cohen's D calculation (Cohen, 1969). The linear mixed effect model was analyzed using the lme4 package (Bates, Maechler, Bolker, & Walker, 2015).

## Results

### Field Study: Temporal pattern of NCEs

### *New Herbivore Pressure* 2016

The probability of documenting beetle damage was reduced only on day 11 (T = -1.99, p = 0.04) and marginally on day 1 (T = 1.88, p = 0.06) (Fig. 2.1). The largest treatment effect on the amount of new herbivore pressure was found in the early season (first 3 site visits) of 2016, where new damage was reduced by 49%. Control and treatment new herbivore pressure in the late season were similar, only 3% less in the treated plots (Fig. 2.2). Overall, treated plots received 22% less new herbivore pressure (T = 1.99, df = 51, p = 0.05), attributed to the early season effect.

### *2017*

The probability of documenting damage and the amount of damage was similar in the treated and control plots (T = 0.91, df = 33, p = 0.37) (Fig. 2.1 and 2.2). The probability of documenting damage was only different on day 18 (T = 2.53, df = 723, p = 0.01).

Adult Beetle Abundance and Ovipostion 2016 & 2017

The average number of adult beetles found on plots throughout both seasons was not significantly different (2016 total adults found on plots- C = 32, T = 27 / 2017- C = 13, T = 28). Despite finding more beetles in 2017 on the treated plots, the difference was not statistically different due to the overall low counts and high degree of variability. Note that since the adult beetles are highly mobile, and there were many other beetles in the field on buffer plants, the plot counts presented here are the conditions at the time of the site visits. Oviposition was not significantly different between the control and treatment in both years (total clutches- 2016, C = 61, T = 48 / 2017, C = 49, T = 45).

Larvae 2016

The average number of larvae found in predator odor treated plots was not reduced by the treatment (T = -1.52, df = 39, p = 0.13), due to the similar numbers of larvae found in the control and treatment plots over the entire season (season total: C = 525, T = 606). However, there were no larvae found in treated plots in the early season between 6/27 – 7/4 (C = 170, T = 0) (Fig. 2.3). Low numbers of *P. maculiventris* were found at the field site and the lack of larvae on the treated plots was likely not due to predation.

2017

The number of larvae was not significantly different (T = -0.21, df = 23, p = 0.83), (total full season larvae- C = 202, T = 304).

Mechanisms of Temporal Patterns in beetle responses to predator odors

Odor Constancy, synthetic predator odor

Volatile dispensers containing *P. maculiventris* pheromone were used to test if changes in the live predator treatment were driving the seasonal pattern in beetle response. Unlike the live predator treatment in either 2016 or 2017, the presence or absence of damage was dictated by the odor treatment over the study period. Plots containing the synthetic odor dispensers were less likely to receive any damage over the season (T = 2.22, df = 2515, p = 0.03) (Fig. 2.4). A marginal season long effect of the treatment on average damage was also found, where treated plots had 21% less damage when compared to the control over the entire study period (T = 1.44, df = 37, p = 0.1) (Fig. 2.5). The season-wide effect suggests that the overwintering and summer beetle generations are responding to the treatment. There was also an effect on the probability of finding larvae on day 13 (T = 2.10, df = 2504, p = 0.04). The number of adults and egg clutches found in plots were not significantly different.

Habituation

Results from the habituation trial did not support the hypothesis that beetles were habituating to predator odors resulting in reduced responsiveness over the season. In contrast to our initial hypothesis, beetles previously exposed to predator odors ate less than naïve beetles (Fig. 2.6). Beetles in the 5- and 24-hour predator pre-exposure treatments consumed similar amounts of plant material. However, beetles that were pre-exposed for 168 hours to predator odors consumed significantly less in the olfactometer than all other combinations ($F_{2, 67} = -2.08$, $p = 0.04$, Cohen's $d = 1.16$).

## Discussion

We found a reduction in plant consumption by *L. decemlineata* in plots that contained live predator odors in 2016, but not 2017. The strongest effect was found in the first three site visits of 2016, where treated plots had 49% less average damage than the control. Furthermore, we observed no larvae in treated plots in the early season of 2016. Two mechanisms were investigated to better understand this temporal pattern of plant damage. We did not find support for some probable hypotheses including beetle habituation or generational differences in responsiveness. There was indirect support for the hypothesis that the predator volatile cue changes over the season. The use of synthetic predator odors in 2017 revealed a more consistent, season-long reduction in the probability of finding plant damage in treated plots, suggesting that the release of P. maculiventris aggregation pheromone varied over the season, influencing the temporal pattern of beetle feeding. Although the seasonal timing of pheromone release from *P. maculiventris* is not known, the duration of attractiveness to their aggregation pheromones is known to be the first 2-3 weeks in spring (Aldrich et al. 1984, Aldrich

1995), approximately coinciding with the early season effect presented here in 2016. This result supports the hypothesis that the higher cue strength and/or a more consistent release in the synthetic predator odor dispensers affected beetle behavior more greatly than the live predator odor treatment.

A potential explanation for the differences in treatment effect between the live predator odor and the synthetic odor in 2017 could be changes in plant quality elicited by the pheromone. Interestingly, some of the compounds in the predator pheromone are also shared with potato plants in the form of green leaf volatiles. One of these compounds, (E)-2-hexenal, has been shown to induce defense-related genes in Arabidopsis (Bate & Rothstein, 1998; Mirabella et al., 2008) and trypsin protease inhibitor production in tobacco upon herbivore damage (Kessler, Halitschke, Diezel, & Baldwin, 2006). The effects of the predator pheromone on potato plant quality is not fully understood and may also contribute, to some degree, to beetle behavioral changes observed here. We are currently investigating the effects of the predator odor compounds on plant quality. Interestingly, recent research has shown that exposing plants to the odors of entomopathogenic nematode-infested cadavers lowered potato plant quality and negatively affected *L. decemlineata* feeding and weight gain (Helms et al., 2019).

Although *L. decemlineata* behavior in the odor treatments did not interact with abiotic measurements, there was a notable disparity in weather patterns in both years. In 2016, the region experienced one of the driest summers on record (Sweet, Wolfe, DeGaetano, & Benner, 2017). In contrast, well above average rainfall occurred during the study period in 2017 (NOAA, 2018). This increase in precipitation also influenced relative humidity. There is evidence that high humidity can have a negative impacts on the responsiveness of invertebrates to volatile chemical cues (Bassett, Baumgartner, Hallett, Hassan, & Symonds, 2011; Hassanali, Nyandat, Obenchain, Otieno, & Galun, 1989; Wilder et al., 2005). For example, Royer and McNeil found that male European corn borers (*Ostrinia nubilalis*) showed a decreased response to pheromone blends as relative humidity increased (Royer & McNeil, 1993). We observed a stronger treatment effect on L. decemlineata feeding in the low humidity of 2016 with the live predator odor and observed no effect in the more humid 2017 season. Furthermore, the more consistent releasing open dispersion dispensers of the synthetic predator odor in 2017 revealed a comparable feeding reduction to the 2016 live predator odor treatment. A better understanding of how increases in relative humidity, as in 2017, and odor cue consistency affect L. decemlineata olfaction and behavior is needed before more comparisons can be made. Future studies using volatile treatments would benefit by incorporating finer-scale weather data, via field deployed sensory networks, at the plot level to find uncover links between the volatile treatment and abiotic conditions.

Kimbro et al. highlighted the importance of investigating NCEs over longer periods (Kimbro, Grabowski, Hughes, Piehler, & White, 2017). By repeating the study and extending the measurement period in this marine system, they found that initially there was a significant response of crabs to predatory toadfish odors, similar to the previous studies (Grabowski, 2004; Grabowski & Kimbro, 2005). However, NCEs cycled over a four-month period from strong to weak to strong again, leading the authors to conclude that predator consumptive effects drive resource dynamics not NCEs. Recent work with L. decemlineata and *P. maculiventris* further illustrates the variable effect of predator cues across different life stages. For example, Wetzel et al. found increased mortality in the presence of predator cues. Interestingly, when these same individuals were tracked through adulthood, overwintering, and spring emergence, it was found that predator exposed beetles had an increased overwintering survival, approximately proportional to the negative effects on the larval stage (Wetzel, Aflitto, & Thaler, 2018). Although we did not measure beetle mortality, the differences in beetle behavior we found across the season as well as interannually support the case for longer periods of inquiry when studying NCEs. Our results also illustrate the potential use of synthetic predatory semiochemicals in agricultural applications.

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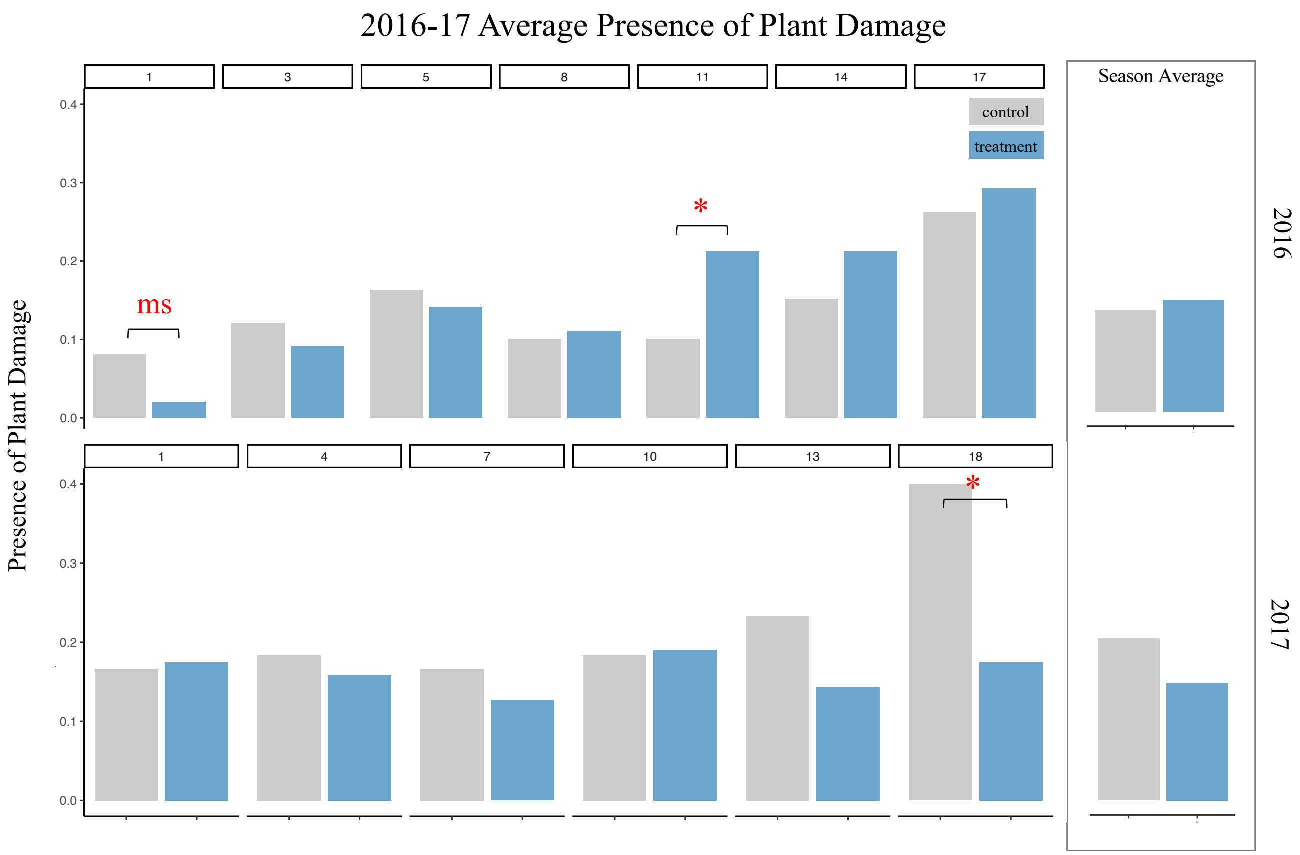

Figure 2.1 The probability of finding damage on a plot. Each set of bars is the average for a given site visit starting on day 1 of the study. Note that error bars were not added due to the Above each set of bars is the day since study start and the right panel shows the season-long average. Asterisks indicate significance; * = p<0.05, ms = p<0.1.

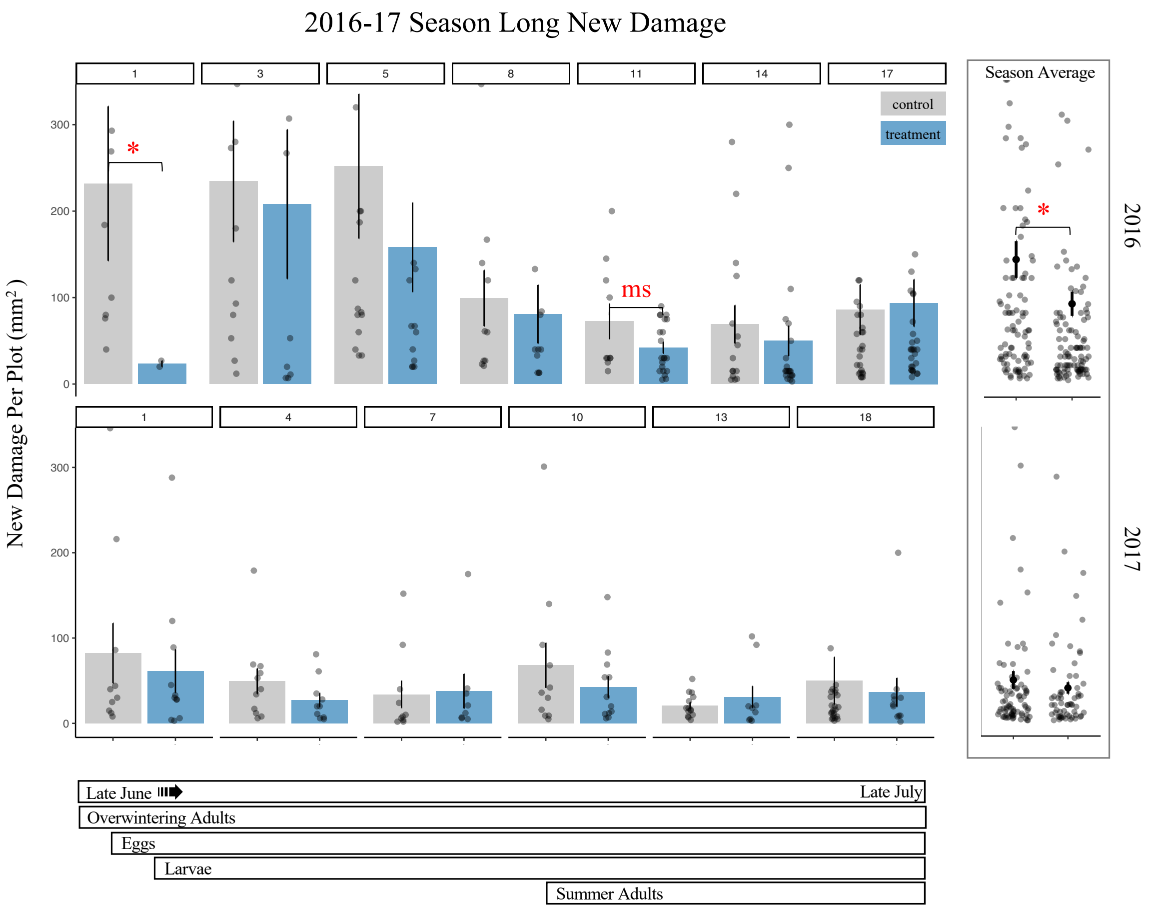

Figure 2.2 New herbivore pressure (proxy for average plot damage) for 2016 and 2017 field seasons. Each set of bars is the average for a given site visit starting on day 1 of the study. Above each set of bars is the day since study start and the right panel shows the season-long average. Bars represent +/- standard error of the mean. The high damage count data points are not shown in this panel for readability purposes. Bottom horizontal bars show the approximate chronological presence of beetle life stages over the study period. Note that overwintering beetles emerge from the soil as adults, and as they oviposit, their offspring become the summer generation. Data presented is not log transformed and zero counts were removed. Asterisks indicate significance; * = $p<0.05$, ms = $p<0.1$.

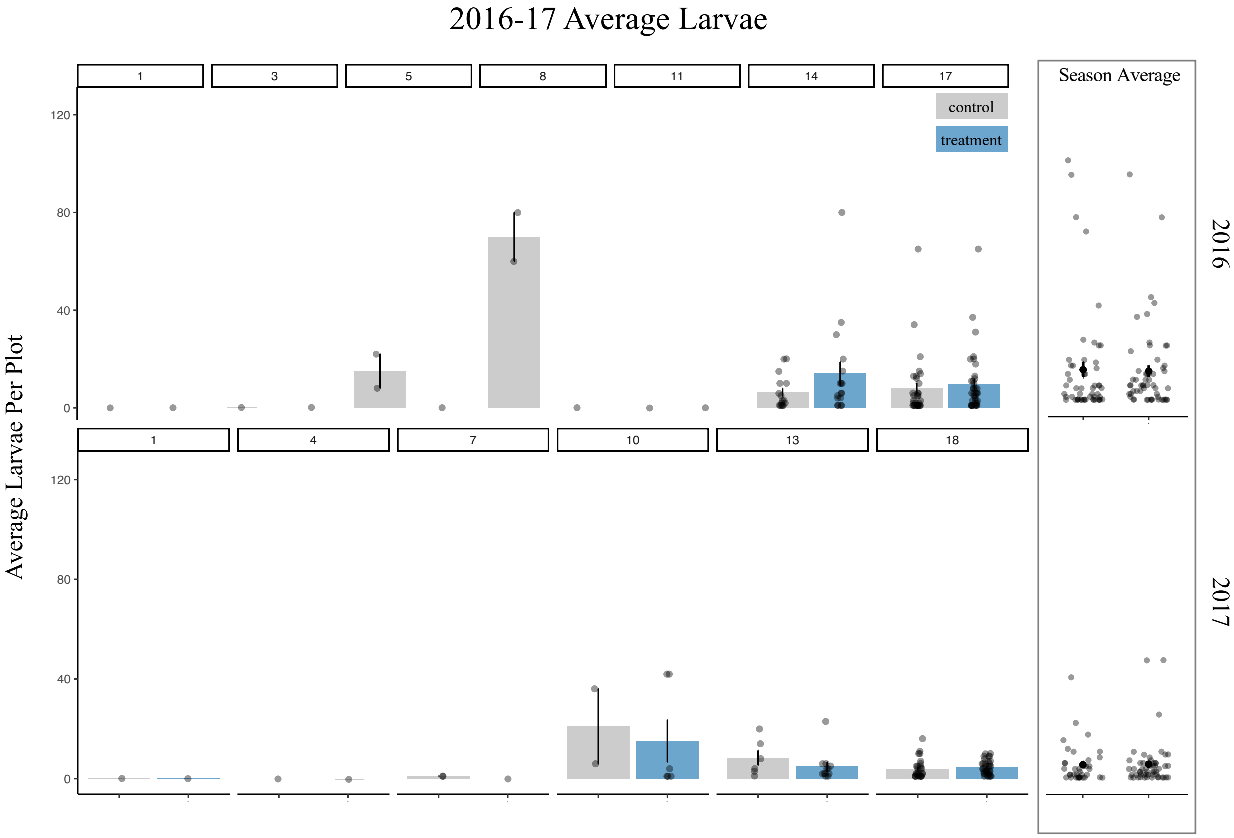

Figure 2.3 Average number of larvae per plot for the 2016 season. Each set of bars is the average for a given site visit starting on day 1 of the study. In total, there were 255 larvae found in control plots in the early season of 2016 and 0 in the treated plots. Above each set of bars is the day since study start and the right panel shows the season-long average. Note, in 2016 on 8 is due to 2 hatching egg clutches. Data presented is not log transformed and zero counts were removed. Bars represent +/- standard error of the mean.

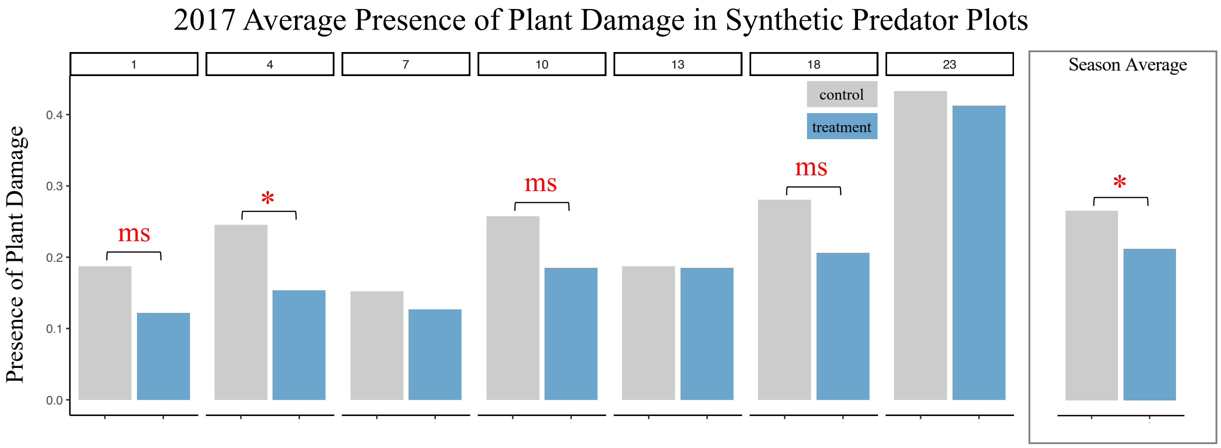

Figure 2.4 The probability of finding damage on a plot. Each set of bars is the average for a given site visit starting on day 1 of the study. Above each set of bars is the day since study start and the right panel shows the season-long average. Asterisks indicate significance; * = $p<0.05$, ms = $p<0.1$.

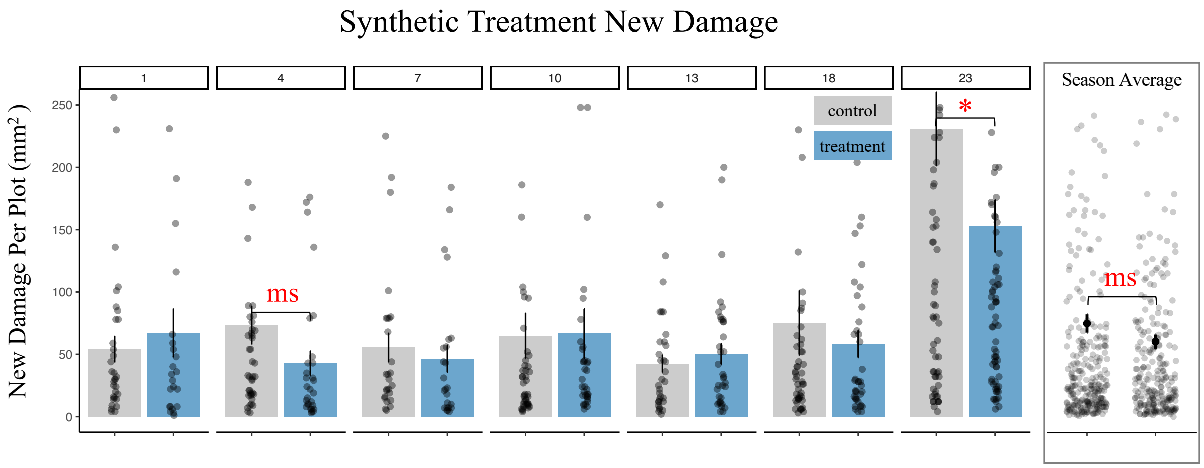

Figure 2.5 Average plot damage for 2017 Synthetic Predator Odor. Each set of bars is the average for a given site visit starting on day 1 of the study. Above each set of bars is the day since study start and the right panel shows the season-long average. Bars represent +/- standard error of the mean. Data presented is not log transformed and zero counts were removed. Asterisks indicate significance; * = $p<0.05$, ms = $p<0.1$.

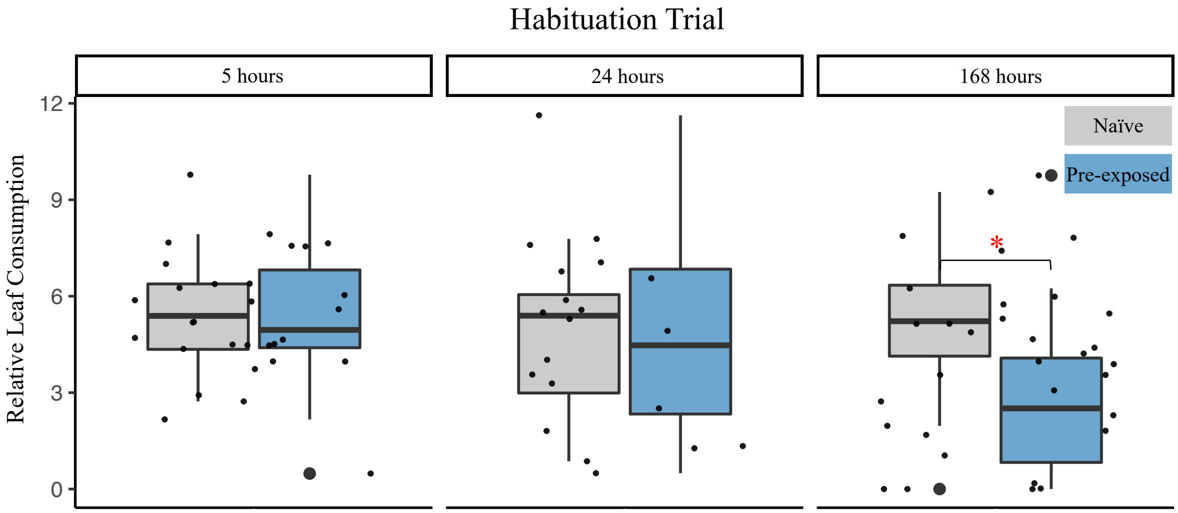

Figure 2.6 Relative plant material consumed in a 24-hour period in the presence of predator odors. The x-axis represents the pre-exposure period of adult beetles to 5 (n=30), 24 (n=20), and 168 (n=29) hours of predator odors prior to running the test. The naïve beetles in the grey bars were not previously exposed to the predator odors. The pre-exposed beetles in the blue bars were the only individuals who previously experienced the predator odors. The y-axis represents adult beetle feeding relative to their initial weight at the study start. Asterisks indicate significance; * = p<0.05.

# CHAPTER 3

# Predator Pheromone Induces Host Plant Defenses and Negatively Affects Prey

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## Abstract

Nearly all prey enact protective measures to reduce the chance of being consumed by predators. Broadly, we have considered the cues responsible for eliciting fear responses to follow a downward linear path, where the cues emanating from higher trophic levels such as predators cascade downward to prey. However, the role of basal trophic levels such as plants and their own ability to sense and respond to their environment has the potential to influence herbivorous prey as well as their predators. In this study, we used lab and field experiments to ask if an insect predator pheromone can alter plant quality and affect the performance of its herbivorous prey. We found that plants exposed to the predator pheromone exhibited several physiological changes such as increased growth and accelerated flowering. The pheromone treatment also affected herbivore prey presence at all life stages and the amount of plant material consumed in field trials. We then investigated to what degree does inducing the plant with the

predator pheromone affect prey behavior compared to directly exposing prey to the predator odors. This test revealed that both direct and indirect pathways are at play, however the greatest effect on prey came from the tests where both pathways are present, reducing plant consumption by 24 percent. The results broaden our understanding of predator-prey interactions by considering a non-linear movement of sensory information, where the cues released from an insect predator change the behavior of a plant, which have negative implications for an herbivore.

## Introduction

The vast majority of animals respond to the sensory cues emanating from their predators by enacting measures that reduce the chance of being consumed, known as non-consumptive effects (NCEs). Although prey response can take many forms such as behavioral, morphological, and physiological (Lima & Dill, 1990; Sih, 1980; Werner & Peacor, 2003), a predominant behavioral response of prey are changes in foraging behavior elicited by predator cues (Lima, 1998; Preisser, Bolnick, & Bernard, 2005; Schmitz, Beckerman, & O’Brien, 1997; Verdolin, 2006). However, such changes in prey foraging behavior often do not explicitly consider the organisms that the prey are foraging on, such as plants, and their own ability to respond to stimuli. Conventionally, NCEs are thought to cascade downward from the predator to prey, however prey response might not always follow a direct path from the predator to the prey. When a predator releases a cue into the environment, it emanates outward for any organism within range and with the appropriate sensory organs to receive. In the insect world, many predator-prey interactions occur on or in close proximity to plants, providing a prime opportunity for plants to intercept the cues released by predators.

Chemical communication via volatile compounds has been shown to be a dominant sensory modality for both plants and insects (Dicke & Sabelis, 1988; Engelberth, Alborn, Schmelz, & Tumlinson, 2004; Johansson & Jones, 2007; Kaissling, 1971; Kessler & Baldwin, 2001; Morrell & Kessler, 2017), often denoting a perceived risk or tissue damage such as alarm semiochemicals in insects (Abassi et al., 2000; Vandermoten, Mescher, Francis, Haubruge, & Verheggen, 2012), green leaf volatiles (GLVs ), and herbivore induced plant volatiles (HIPVs) in plants (Engelberth et al., 2004; Farmer & Ryan, 1990; Schultz, 2002). In addition to conspecifics receiving these volatile signals, a variety of other organisms within perceptual range may receive and exploit the information. Green leaf volatiles, which are C6 molecules rapidly produced by plants after damage, are known to be an important source of information for herbivores (Visser & Avé, 1978), predators (Dickens, 1999), and parasitoids (Whitman & Eller, 1990). However, a plant's response to sources of volatile chemical signals from predatory trophic levels has received little consideration despite an abundance of literature illustrating the ability of plants to perceive such cues. With the exception of belowground cues released from entomopathogenic nematodes (An, Orellana, Phelan, Cañas, & Grewal, 2016; Helms et al., 2019), the use of third trophic level cues by plants has not been well characterized. An alternative theory to observed changes in prey foraging behavior when predator cues are present are changes in plant quality that are elicited by cues from the predator, making host material less palatable to herbivores (Fig. 4.1).

This shared sensory channel between the predator and the plant solicits intriguing questions, such as: Are plants able to detect and respond to above ground predator odor cues? If so, is there an advantage to plants by detecting the presence of a predator via the elicitation of indirect NCEs (INCEs)? To test the indirect NCE hypothesis, we used a well-studied system comprised of *Leptinotarsa decemlineata* (Colorado potato beetle) as the prey herbivore and the volatile aggregation pheromone of the generalist predator, *Podisus maculiventris* (spined soldier bug). First, we administered the predator pheromone of the *P. maculiventris* in a potato field and measured the behavior of *L. decemlineata* over a growing season. Next, we used a series of lab studies to test the hypothesis that plants are inducing defensive compounds after being pre-exposed to the predator pheromone. After confirming that *L. decemlineata* feeds less on plants that were pre-exposed to the predator pheromone, we conducted a phytohormone analysis to understand the changes occurring within the plant. We then used a factorial design to learn to what degree do the indirect and direct pathways ultimately contribute to the prey behavioral change.

## Methods

### *Field Study*

On May 9, 2018, approx. 10,000 *Solanum tuberosum* (cv Yukon Gold and Superior) potatoes were planted at Cornell University's Homer C. Thompson Vegetable Research Farm in Freeville, NY. Previous research in this system has used Yukon Gold and the addition of another early-season potato variety, Superior, was used to broaden our understanding of the effect. Ultimately, since the results were similar in both varieties, they were combined for the analyses. Prior to planting, the site was fertilized with Triple 13 NPK at a rate of 1289 kg/ha. Seeds were planted 0.3m apart within rows with row spacing at 1m apart. Following standard potato planting practices, the maximum number of rows in a block did not exceed 14 to allow for the movement of equipment in the field site. The site was inspected for *L. decemlineata* adults and eggs and defaunated daily after the first emergence of plants. Once plants reached approx. 20 cm in height on June 6th and 7th, 2018, 200 (93 Yukon Gold, 107 Superior) plots were installed consisting of 3 plants each. Treated plots received an open dispersion volatile dispenser made from a modified opaque brown 2 mL Eppendorf tube with 3 0.55 mm holes in the top. Each dispenser was filled with 594.1µL of a synthetic male *P. maculiventris* dorsal abdominal gland pheromone mixture (354.0 µL (*E*)-2-hexenal, 216.0 µL alpha terpineol, 2.4 µL linalool, 2.0 µL terpinen-4-ol, and 19.7 µL benzyl alcohol) (Aldrich, Kochansky, & Abrams, 1984) and affixed to a flag at the plot center. The dispensers emitted the pheromone at an approx. average rate of 0.23 mg/hr. Dispensers remained in the field for the measurement period and were replaced every 2 weeks. Others have found that a single male *P. maculiventris* can release up to a maximum of 1 mg of the pheromone at a time (Aldrich et al., 1984). Further, other studies have used live *P. maculiventris* as an semiochemical source showing a reduction plant consumption by *L. decemlineata* (Aflitto & Thaler, 2020; Hermann & Thaler, 2014), yet changes in plant behavior have not been previously measured. Control plots received empty Eppendorf tubes. Treatments were randomized across both varieties.

To facilitate Colorado potato beetle colonization, beetles were collected from potted trap plants around the farm and maintained in a lab colony on Yukon Gold potatoes in a 16:8 L:D cycle. After the treatments were installed, 600 field-collected beetles were released at 10 evenly spaced intervals down each of the rows. Beetles were then allowed 24 hours to establish before measurements began.

Five measurements were taken during every site visit on each of the 3 plants: number of adult beetles, larvae, egg clutches and number of eggs per clutch, and the amount of new herbivore pressure (herbivory proxy). New herbivore pressure was measured on one focal leaf per plant. The focal leaf was marked by a small piece of flagging, which was moved to an undamaged leaf each site visit. This prevented double counting damage and avoided instances where the leaf was fully consumed, thus not measurable. The amount of leaf tissue removed was measured with a 2 mm x 2 mm grid printed on clear acetate. In total, five site visits between June 8 and July 7 were completed.

### *Lab Studies*

#### *Pre-Exposure Induction Test-*

Plants: Yukon Gold plants were grown in 4-inch plots in a greenhouse until they reached approx. 20cm in height. All study plants were in the vegetative state. Once plants were of suitable size, they were enclosed inside of a plastic bag (22.9 x 30.5cm, 1.5 mil, Elkay Plastics) containing a rubber septum (5 x 9 mm) loaded with 250 µL of a neat predator pheromone blend (same as field formulation) or a control septum. After completing the neat blend test, additional tests with a diluted 1% blend (1 percent pheromone: 99 percent hexane) were tested against control septum charged with pure hexane. In the 1 percent tests, we only enclosed a leaf of the plant and left the remainder of the plant out of the pre-exposure bag. Our goal by assaying both inside and outside of the treatment bag was to better understand the systemic response in the plants. The bags were carefully sealed around the leaf stem using a wire tie and exposed to the compounds for 72 hours in a growth chamber at 26° C for a 16:8 light cycle. At the end of the pre-exposure period the bags were removed, and the plants were allowed to rest for 12 hours. Once the resting period was over, terminal leaflets were removed from the plants for a bioassay.

### *Bioassay*

By allowing the larvae to feed in the absence of the predator pheromone, our goal was to target the plant's influence on the herbivore, or the indirect NCE rather than the direct NCE. Each of the excised pre-exposed leaflets were inserted into water picks and placed inside of a 240 mL clear plastic cups with breathable mesh lids. Neonate larvae less than 48 hour old were weighed and placed on leaflets and allowed to feed for 72 hours. Larvae were collected from a lab colony of *L. decemlineata* maintained on 16: 8 L:D dark cycle and fed on Yukon Gold potato plants. This assay was repeated on 2 different dates for the neat pheromone blend, and 5 dates for the 1 percent.

### *Phytohormone Analysis*

Leaf samples weighing 200-300 mg were carefully excised from plants using a clean razor blade and placed into a 2 mL screw top vial. The 4th expanded leaf was excised for analysis. Each vial was then immediately placed in liquid nitrogen until transferred to -80° C for storage. Leaf samples were then lyophilized, reweighed, ground in a Harbil 5G-HD paint shaker, and extracted according to Casteel et al., 2015. Each sample was spiked with isotopically labeled hormone standards prior to extraction. All samples were run on a Thermo Scientific Q Exactive Hybrid Quadrupole-Orbitrap HPLC at the Boyce Thompson Institute. In total, 6 control and 6 treatment samples were used in the analysis.

*Direct NCE v. Indirect NCE-*

In order to quantify the pathways that contribute to the beetle's behavioral change, the presence of the predator pheromone was crossed in a fully factorial design with plants that were pre-exposed to the pheromone. Plants: Yukon Gold plants were grown in 4-inch pots in a greenhouse until they reached approx. 20 cm in height. As in the previous lab study, only plants in the vegetative stage were used. Once plants were of suitable size, they were pre-exposed using the same methods as the pre-exposure induction test, half receiving the hexane control treatment (n=30) and half the 1 percent pheromone blend (n=31).

*Bioassay*

After 3 days of pre-exposure, and a 12-hour resting period, the plants were split into 4 treatment groups for the bioassay: 1) Control plants with no pheromone (250 µL hexane septa), 2) Control plants with pheromone (250 μL 1 percent blend septa), 3) Pre-exposed plants with no pheromone (250 μL hexane septa), 4) Pre-exposed plants with pheromone (250 μL 1 percent blend septa). One terminal leaflet of similar age from each plant was used and placed in the same water pick and cup assay system as the pre-exposure induction test. Larvae were allowed to feed, and consumption was measured at hours 2.5, 9, 24 and 48, repeated on 5 different dates. Consumption measurements were made relative to the larval weight at the start of the assay [RC = consumption (mm) ÷ larval weight (mg)]. Efficiency of conversion of ingested leaf tissue was calculated using the following formula [ECI = weight gain (mg) ÷ consumption (mm)]. Plant flowering stage was also measured from this assay at 3 and 7 days post initial exposure to the pheromone and rated on a scale from 0 - 5 (0 represented a pre-flowering plant - 5 represented a fully expanded flower).

### *Statistics*:

#### Field Study

Plant damage, number of adults, egg clutches and larvae were analyzed using mixed models. Hurdle models were used due to the large number of observed zero values in the response variables. A binomial generalized linear mixed model was used to model the probability of the presence of each response variable individually (plant damage, adult count, egg clutch count, or larval count). In each model, treatment was included as a fixed effect and plant nested within plot and sample date were included as random effects. A linear mixed model was used to model the effect of the treatment on the non-zero amount of average plot damage and larval abundance. In each model, treatment was included as fixed effects and plant nested within plot and sample date were included as random effects. Average plot damage and average larvae were square root transformed to meet the linear model assumptions of normality and homogeneous variance. Note, the data presented in the figures are not transformed. Linear models were not fit to the adult colonization and oviposition data due to the low number of non-zero values.

### Lab Studies

Larval leaf damage and ECI were analyzed with mixed models. In each model, relative leaf damage or ECI and treatment were fixed effects and parental genetics and the bioassay date as random effects. Leaf consumption was made relative to the initial starting weight of the larvae and were square root transformed to meet model assumptions. Phytohormones were analyzed using ANOVA. Flowering stage was analyzed using a linear mixed model with flower development rating and time since exposure as fixed effects and date nested within experiment run as a random effect.

All analyses were completed using R statistical software (R Core Team 2014). Mixed models were analyzed using the lme4 package (Bates, Maechler, Bolker, & Walker, 2015).

## Results

### *Field Study*

### *Effect of predator pheromone on beetle presence, plant damage, and yield*

Since overall adult and oviposition counts were low, only a binomial GLM was used to fit the adult and egg data. The probability of finding an adult beetle was 64% lower in the pheromone treated plots over the season ($Z = -2.39$, $p = 0.017$) (Fig. 3.2) in comparison to the control plots. Total beetle counts in pheromone treated plots were lower over the season in both varieties (C = 60, T = 20). There was a reduced probability of finding an egg clutch in the pheromone treatment plots ($Z = -2.52$, $p = 0.012$) (Fig. 3.2). The total number of eggs laid on a plot was lower in the treated plots ($T = -2.72$, $p = 0.007$) in comparison to the untreated plots. The probability of finding larvae on plots was marginally influenced by the treatment ($T = -1.72$, $p = 0.086$) (Fig. 3.2).

Since larval counts and plant damage were high unlike the adult and egg data, linear mixed effects models were also used to analyze average number of larvae and amount of damage per plot. The average number of larvae per plot was reduced by the treatment by 31% ($T = -2.13$, $p = 0.031$) (Fig. 3.3). There was also a significant effect of the treatment on the average amount of damage ($T = 8.68$, $p = 0.004$), a reduction of 46% (Fig. 3.3).

There was no effect on yield, however the pheromone treated plots trended 14% higher ($T = 1.62$, $p = 0.11$) (Fig. 3.4).

*Lab Studies*

*Pre-Exposure Induction Test-*

Leaves from inside of the pre-exposure bag (local induction) and leaves from outside of the bag (systemic induction) were assayed to better understand the plant response. Overall, plants that were pre-exposed to the neat pheromone ($T = 2.40$, p = 0.02) and the 1 percent dilution ($T = -2.93$, p = 0.004) (Fig. 3.5) exhibited reduced beetle consumption in feeding assays for the leaves that were inside of the pre-exposure bag. The systemic induction test using leaves from outside of the pre-exposure bag also revealed a marginally significant difference in consumption ($T = 1.189$, p = 0.06) (Fig. 3.5).

*Phytohormones-*

Jasmonic acid (JA) trended 49 percent higher in plants that were pre-exposed to the pheromone, however was not statistically significant ($F = 1.47$, df = 11, p = 0.2). Salicylic acid (SA), 1-aminocyclopropane-1-carboxylic acid (ACC), and abscisic acid (ABA) were all similar in the treatment and control plants (Fig. 3.6).

*Direct v. Indirect Trial-*

There was no difference in consumption at 2.5 hours after starting the bioassay. At 9 hours, a marginally significant reduction in consumption in the control leaf + predator pheromone group was found ($T = -1.51$, df = 126, p = 0.1) (Fig. 3.7). At 24 hours, the treatment effects become more apparent, with the treatment leaf + predator pheromone showing the greatest reduction ($T = -3.05$, df = 100, p = 0.003), followed by the control leaf + predator pheromone ($T = -1.92$, df = 100, p = 0.06). At 48 hours, reduced consumption was not significantly different, however the efficiency of conversion of ingested leaf tissue (ECI) was significantly higher for all of the treatments (control + pheromone- $T = 2.44$, df = 30, p = 0.02 / treatment leaf- $T = 2.15$, df = 30, p = 0.03 / treatment leaf + pheromone- $T = 2.21$, df = 30, p = 0.04). We also found that after the 3-day pre-exposure period up to 7 days post exposure, exposed plants began flowering earlier than the control plants ($T = 2.26$, df = 193, p = 0.02) (Fig. 3.8).

## Discussion

By pre-exposing plants to the *P. maculiventris* predator pheromone, we were able to highlight an alternative flow of information where aerial predator cues elicit changes in prey resources (plants) that in turn have a negative effect on prey performance. We found a clear benefit to the plant in responding to the predator pheromone by having less damage by herbivores in the field and lab, and fewer *L. decemlineata* adults, larvae, and egg clutches in the field study in large part due to induced plant responses. The field results also highlight the potential deterrent effect of the pheromone as we documented a 64 percent reduction in the presence of adults and a lower average number of larvae on treated plots. Logically, finding fewer beetles on a plot should lead to lower overall plant damage, and we found a 46 percent damage reduction. In the no-choice lab studies, we documented an average reduction of approx. 28 percent. The difference between field and lab damage reduction might be explained in part by the free choice of *L. decemlineata* in the field.

There were no differences in phytohormone levels between plants that were pre-exposed to the pheromone and control plants. However, JA trended 49 percent higher in pre-exposed plants and might help explain the herbivore's response we measured. Jasmonic acid is typically wound-induced, activating defenses against herbivores such as protease inhibitors (Farmer & Ryan, 1990; Green & Ryan, 1973; Thaler, Farag, Paré, & Dicke, 2002). Jasmonic acid can also be activated through exposure to green leaf volatiles (GLVs) (Hirao et al., 2012). Although the mechanisms behind *L. decemlineata* response to the pheromone-treated plants remains to be fully understood, a probable explanation for the plant response to the predator pheromone may be the interpretation of some of the pheromone components by the plant as GLV signaling compounds (Aflitto & Thaler, 2021). One prime suspect is (*E*)-2-hexenal, which makes up approximately half of the predator pheromone blend (Aflitto et. al, in prep), and has been shown to be important in plant-plant communication (Arimura, Ozawa, Horiuchi, Nishioka, & Takabayashi, 2001; Hirao et al., 2012; Kishimoto, Matsui, Ozawa, & Takabayashi, 2008). (*E*)-2-hexenal, which is found in nearly all green plants, is one of the first signaling compounds to be released aerially from damaged tissue (Farag & Paré, 2002). Once in the atmosphere, distal parts of the same plant and neighboring plants can receive the signal and prepare chemical defenses for a potential herbivore attack. Current research is underway to understand if the predator pheromone is being misinterpreted by the plant as a green leaf signal or if the plant is receiving this cue as an honest signal from the stink bug.

We also observed changes in flowering in pre-exposed plants, which may indicate hormonal changes. For example, JA is known to be involved in numerous physiological functions such as flowering (Browse, 2009; Wasternack, Forner, Strnad,

& Hause, 2013). Although we did not find that pheromone-exposed plants had higher levels of JA, the results trended 49 percent higher in the pre-exposed plants. Others have found that when plants are exposed to defense elicitors such as the volatile ester of JA, methyl jasmonate, defensive compounds are primed not activated. After the pre-exposed leaves received damage, defensive compounds were detected (Kessler, Halitschke, Diezel, & Baldwin, 2006). More research is needed to investigate the effects of a feeding herbivore on phytohormone levels in pheromone pre-exposed plants, as our analysis was only conducted on undamaged leaves.

The notion that organisms are linked through a hierarchical order has framed our understanding of ecological systems, leading to concepts such as trophic cascades (Elton, 1927; Paine, 1980; Price et al., 1980). The way we consider information transfer in predator-prey interactions has generally followed this framework, where predators elicit a prey response and prey anti-predation behavior affects downstream trophic levels (Abrams, 1995; Beckerman, Uriarte, & Schmitz, 1997; Carpenter, Kitchell, & Hodgson, 1985; Menge, 1995; Power, Marks, & Parker, 1992; Schmitz et al., 1997; Schmitz, Krivan, & Ovadia, 2004; Trussell, Ewanchuk, & Matassa, 2006). However there are exceptions, such as cases where predators are attracted to herbivore induced volatiles (HIPVs) in the hopes of locating prey (Allison & Daniel Hare, 2009; De Moraes, Lewis, Pare, Alborn, & Tumiinson, 1998; Rodriguez-Saona, Kaplan, Braasch, Chinnasamy, & Williams, 2011; Thaler, 1999) and plants responding to cues from belowground natural enemies (An *et al.*, 2016; Helms *et al.*, 2019). The work presented here considers a terrestrial tri-trophic interaction, however the same indirect interactions are likely to occur in other systems. The omni-directional nature of how information moves in the environment might not always modify interactions in predictable ways. To better understand the complex sensory ecology of species interactions, a holistic perspective on information transfer in the complete system, not simply the organisms under study, will reveal novel interactions and methods to alter the behavior of organisms for management purposes.

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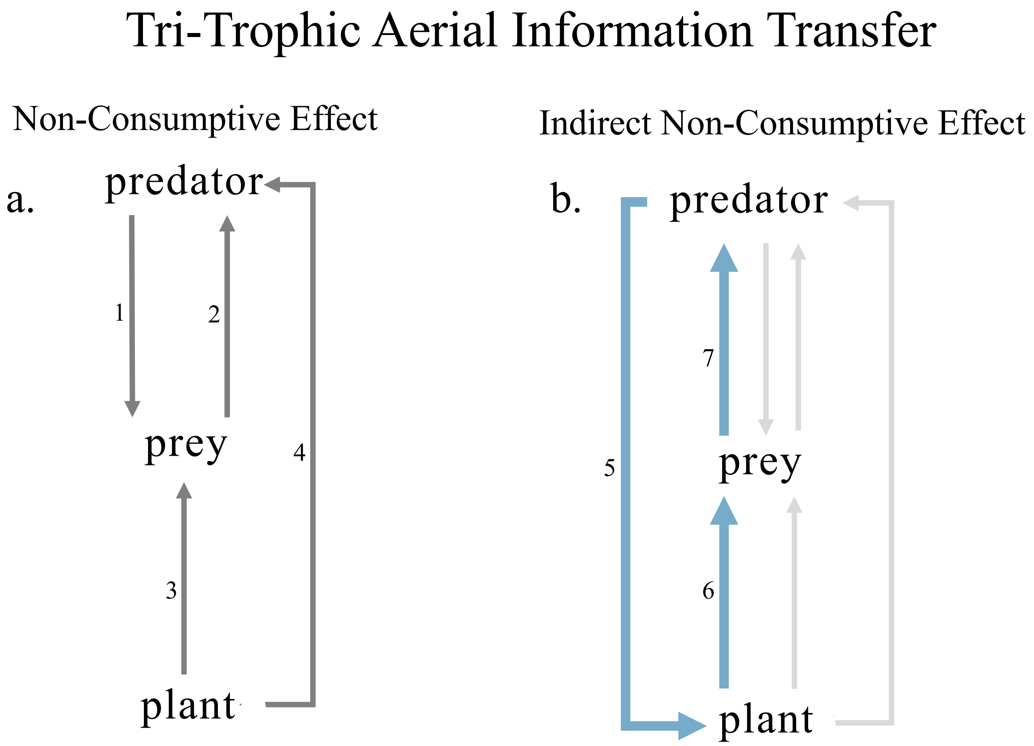

Figure 3.1 Conceptual comparison of the semiochemical information transfer of (a) non-consumptive and (b) indirect non-consumptive effects we present in this paper. (a.1, 2) predator and prey detect each other's signals and (a.3, 4) predator and prey detect plant signals, encompassing the conventional flow of aerial information in a tri-trophic predator-prey interaction. The blue arrows in panel b. represent an alternative flow of information, where (b. 5) the plant detects and responds to a signal from a predator, (b. 6) having an effect on the prey. The effect on the predator is not yet resolved (b. 7).

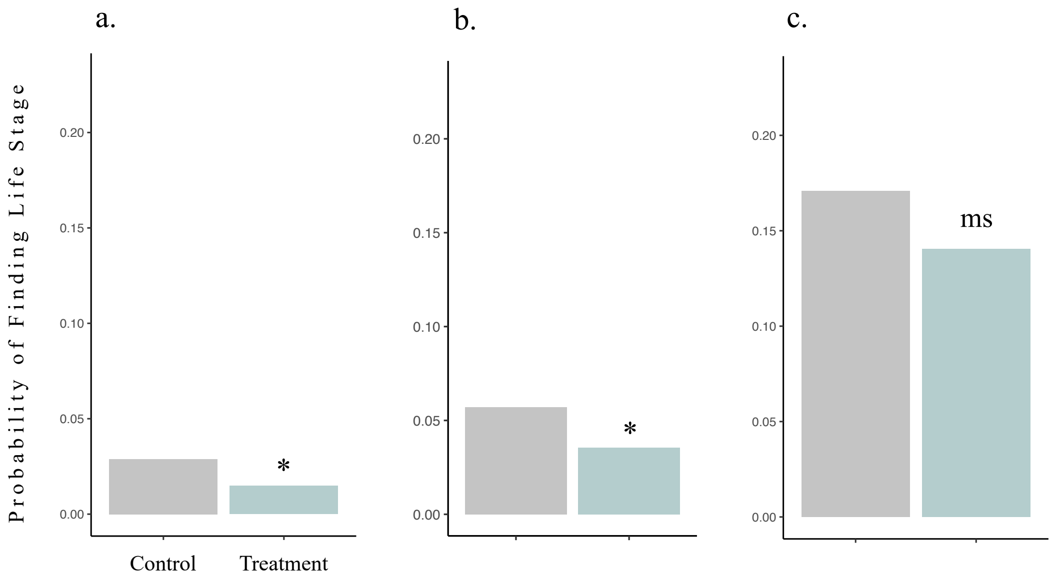

Figure 3.2 The probability of finding *L. decemlineata* life stages; (a.) adult beetles, (b.) egg clutches, and (c.) larvae on control and *P. maculiventris* predator pheromone-treated field plots over the season. Asterisks denote a p-value < 0.05.

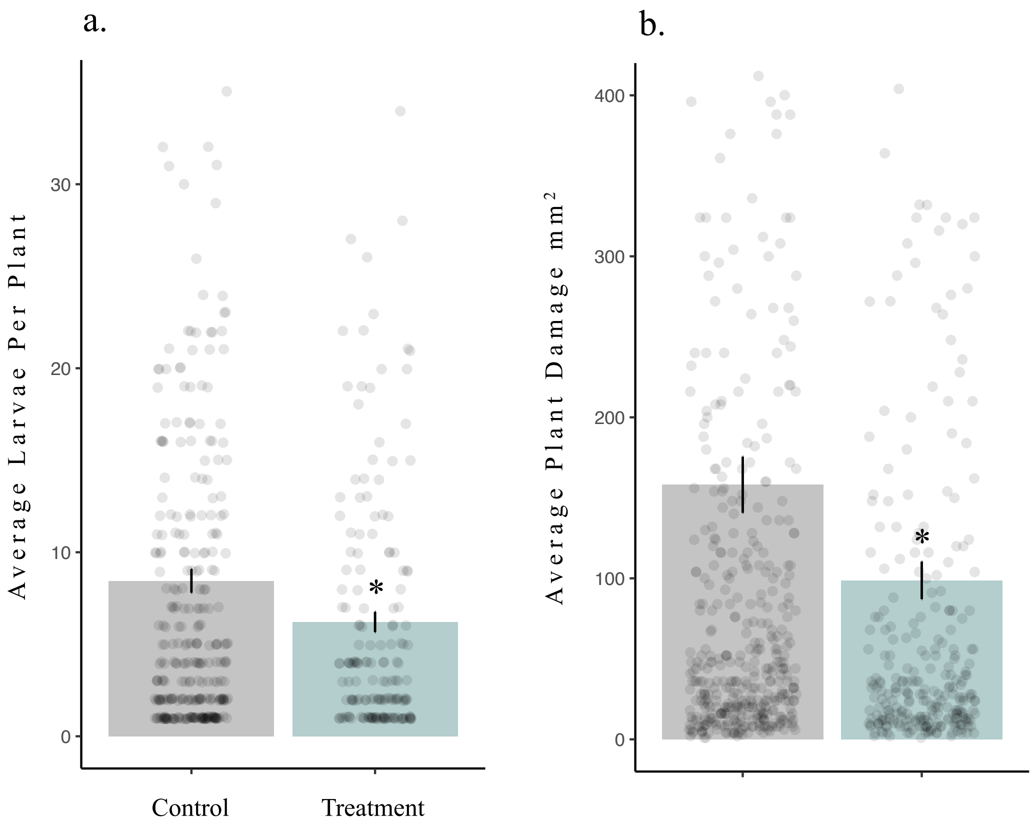

Figure 3.3 The mean number of *L. decemlineata* (a.) larvae and (b.) plant damage found on *P. maculiventris* predator pheromone-treated field plots over the season. Bars represent the standard error of the mean. Asterisks denote a p-value < 0.05.

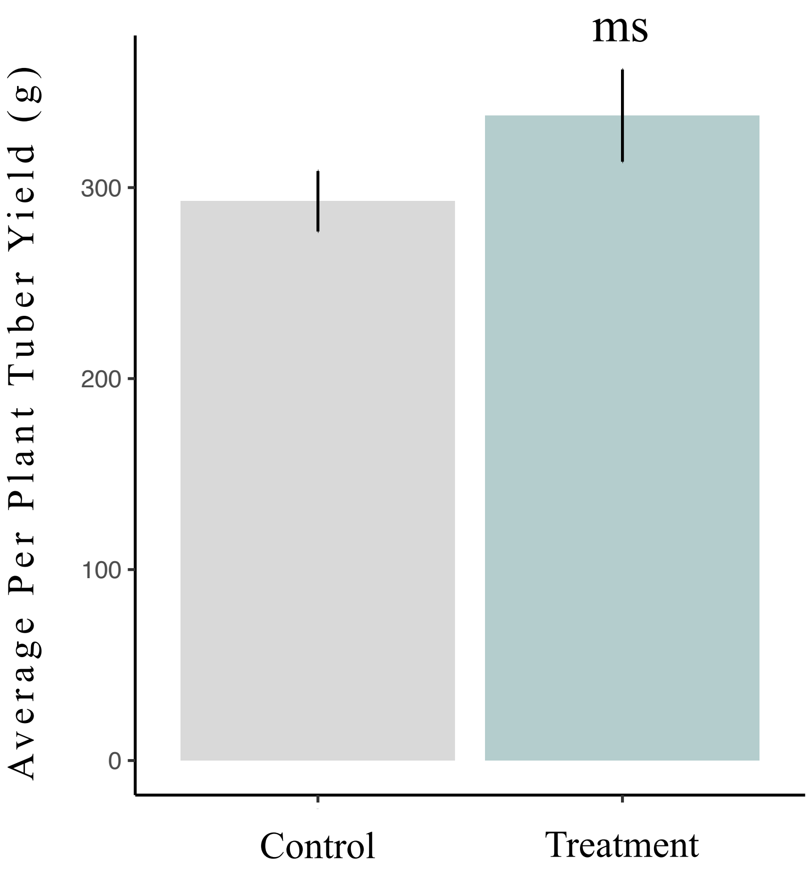

Figure 3.4 Mean *S. tuberosum* total tuber yield in grams at the end of the season in the control and *P. maculiventris* predator pheromone-treated plots. Bars represent the standard error of the mean.

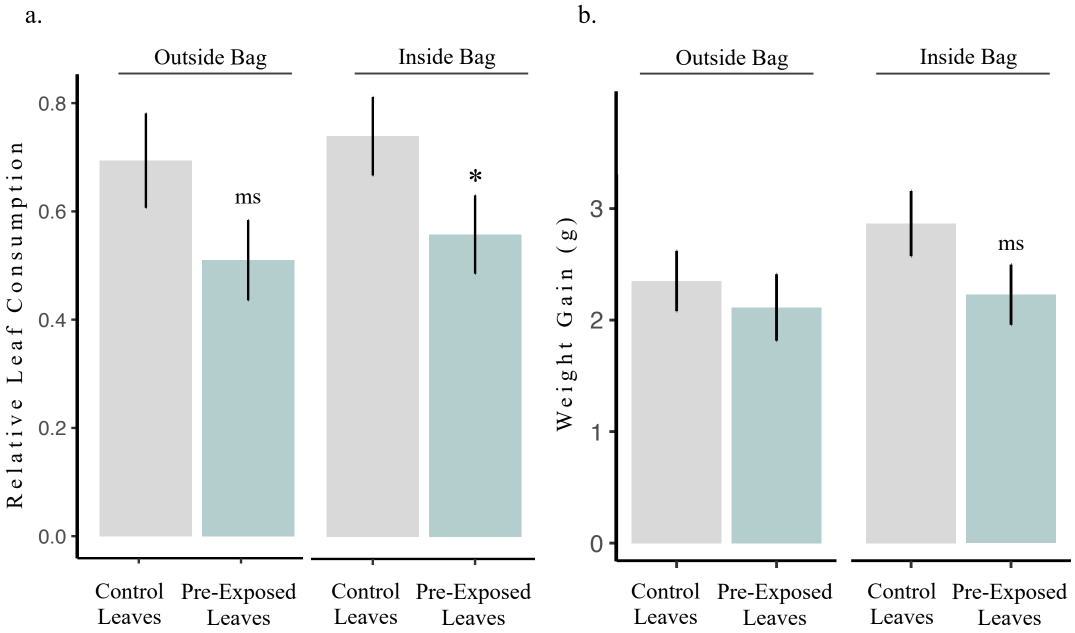

Figure 3.5 (a.) Mean relative consumption and (b.) mean weight gain of neonate larvae for the 1% diluted *P. maculiventris* predator pheromone plant pre-exposure. Potato leaves were assayed outside (Outside Bag) and inside (Inside Bag) of the treatment headspace bag. All of the consumption measurements were made relative to the starting weight of the *L. decemlineata* larvae at the beginning of the trial. Note, the undiluted pheromone blend trials followed the same trend, but are not displayed here. Bars represent the standard error of the mean. Asterisk denotes a p-value < 0.05.

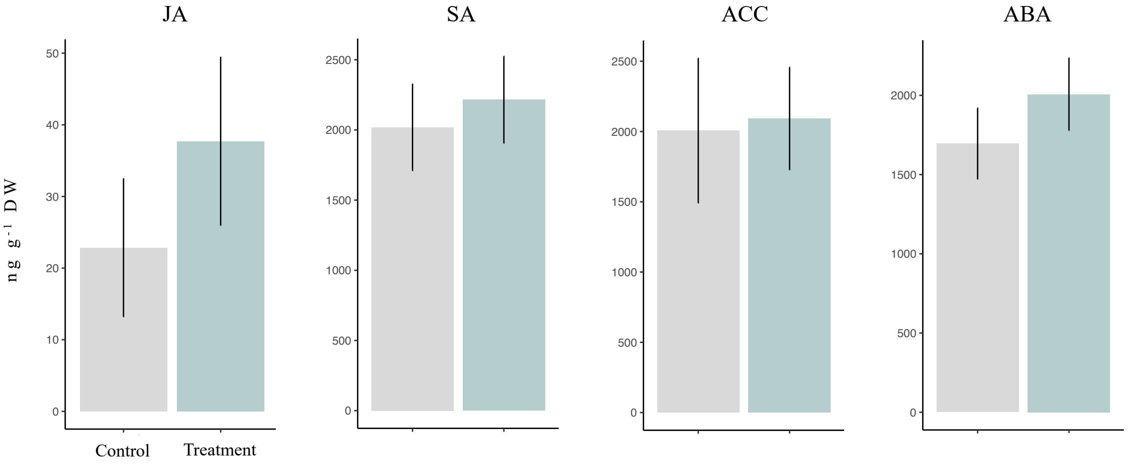

Figure 3.6 Mean concentrations of phytohormones in potato plants that were pre-exposed to the *P. maculiventris* predator pheromone for 3-days. DW = dry weight. Bars represent the standard error of the mean. Comparisons were determined using ANOVA.

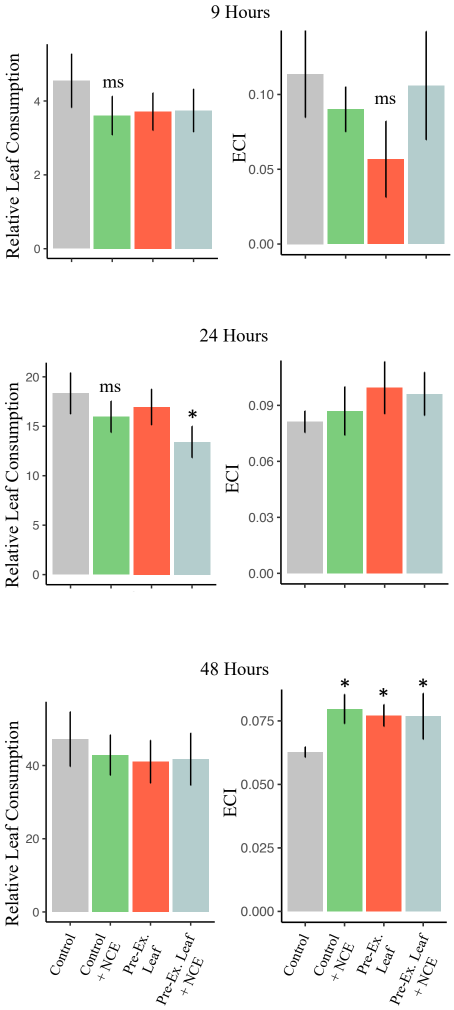

Figure 3.7 Mean relative consumption and efficiency of conversion of ingested leaf tissue (ECI) for 9-, 24-, and 48-hour check points of *L. decemlineata* larvae. The control + NCE treatment had the presence of the *P. maculiventris* predator pheromone. The pre-exposed leaf had only been previously exposed to the pheromone for 3-day and no pheromone present during the assay. The pre-exposed leaf + NCE had pheromone present in the assay and the leaves were previously exposed. Note the different y-axis scales needed as beetles grew. Bars represent standard error. Asterisks denote a p-value < 0.05.

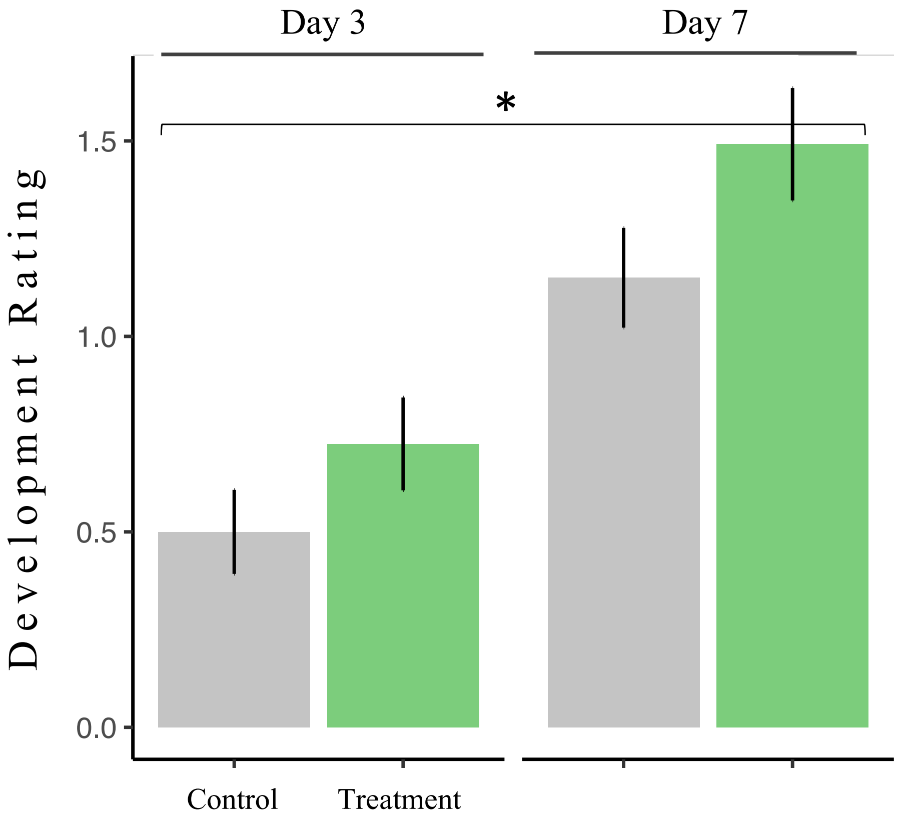

Figure 3.8 Floral developmental stage from 80 *S. tuberosum* plants measured at 3- and 7-days post *P. maculiventris* predator pheromone pre-exposure. Measurement taken on a 1-5 scale further described in the methods. Bars represent the standard error of the mean. Asterisks denote a p-value < 0.05.

## CHAPTER 4

# Shared semiochemicals in a tri-trophic system benefit crop plants†

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**Abstract-**

Prey commonly use volatile chemicals released from predators to infer the level of danger and can enact phenotypic changes to increase their chance of survival. Because some predators emit volatiles that are also used in plant defense signaling, there is the potential for plants to also respond to predator cues. In the Colorado potato beetle, *Leptinotarsa decemlineata*, predator spined soldier bug, *Podisus maculiventris*, potato, *Solanum tuberosum*, system, the beetle responds to the predator aggregation semiochemical, which is comprised of predator-specific compounds and compounds that are known to be used by plants as green leaf volatile signals to induce their own defenses. Given this shared sensory cue in the system we asked the question; is the effect of the predator semiochemical on prey driven by the full predator semiochemical, or are there bioactive compounds in the blend that are also shared with the plants that are responsible for the prey behavioral changes? By fractionating the semiochemical into three treatments (full blend, shared cues, and predator only) and dispensing it in a replicated potato field with free-ranging herbivores, we found that the cues shared with the plant reduced herbivore feeding by 37 percent and the full blend by 41 percent compared to the control or predator specific fraction. Potato plants also responded to the shared cues by growing larger over the season and initiating flowering earlier, indicating that prey responses to the semiochemical could be direct or mediated by the plant's response to the semiochemical. These findings highlight the potential utility of using shared cues for management purposes. Rather than using a semiochemical treatment that has a single target audience (i.e., the pest), we showed that a cue shared across multiple trophic levels decreased plant damage and increased growth, while eliciting anti-predation behavior in the prey.

**Key Words-** applied chemical ecology, integrated pest management (IPM), non-consumptive effects (NCE), semiochemicals, sensory ecology, trait-mediated indirect interactions (TMII), tritrophic interactions.

## INTRODUCTION

The world is rife with sensory information and organisms are challenged with the task of sifting through all of these cues to make rapid judgements. The vast number of cue combinations possible across numerous modalities gives the impression that each signal is highly specific, and in many cases that is true, as in private channel communication (Raguso, 2008). Conversely, there are certain cues with widespread distribution in the natural and managed systems that carry useful information for multiple trophic levels, setting up a situation where numerous organisms in a system detect and respond to the same cue, often for different purposes (Lee, Wood, & Lee, 2015; Tomberlin et al., 2017).

There is the potential for information overlap in systems when numerous organisms make use of and produce the same cues. Although there are many differences in the perceptive abilities of plants and animals, chemoreception is an essential sensory modality in both. Many insects, including predatory Heteropterans, release a plethora of volatile compounds, many of which are the same compounds found in plant green leaf volatile (GLV) signals. For example, plant-produced GLV compounds like (*E*)-2-hexenal are also released and used by numerous animals (Jeffrey R. Aldrich, 1985; Blatt, Borden, Pierce, Gries, & Gries, 1998; Gries et al., 2015; Lockwood & Story, 1987) and at the time of writing this, (*E*)-2-hexenal is known to be a part of the communication systems of at least 209 insects, 62 of which are Pentatomid stink bugs (found in Pherobase, Dec. 2020).

In addition to other organisms releasing GLV-shared compounds, many organisms detect and make use of plant GLVs (Turlings et al., 1995; Visser & Avé, 1978; Webster & Carde, 2017). Since plants are sessile and cannot avoid attackers like motile organisms, one of their first lines of defense upon attack is the release of GLVs from damaged leaf tissue (Farag & Paré, 2002; Szafranek & Szafranek, 2008; Visser, Straten, & Maarse, 1979). The initial surge of GLVs after biotic stress consists of a single compound class, hexenals such as (*E*)-2-hexenal (Ameye et al., 2018). When plants receive an airborne GLV signal, secondary chemical defenses can be primed in preparation for an attack by an herbivore. Green leaf volatiles are also an important source of information for herbivores and predators for host identification purposes (Kessler & Baldwin, 2001; Schütz, Weißbecker, Klein, & Hummel, 1997).

In this study, we used a system involving a generalist Pentatomid predator (*Podisus maculiventris,* spined soldier bug), potatoes (*Solanum tuberosum*, cv Yukon Gold), and a common herbivorous insect (*Leptinotarsa decemlineata,* Colorado potato beetle). All three trophic levels under study are known to detect (*E*)-2-hexenal (De Jong & Visser, 1988; Dickens, 1999; Farag & Paré, 2002; Weißbecker, Schütz, Klein, & Hummel, 1997), and the predator (J. R. Aldrich, Kochansky, & Abrams, 1984) and plant (Visser & Avé, 1978) are both known to release large quantities of (*E*)-2-hexenal. Further, the extremely common plant terpenoid linalool is also released by potato plants and the predator as a semiochemical, albeit at much lower volume than (*E*)-2-hexenal (Jeffrey R. Aldrich, Kochansky, Lusby, & Sexton, 1984). *L. decemlineata* has receptors that detect linalool (Weißbecker et al., 1997). In this system, it has been shown that the presence of the predator semiochemical cue causes the *L. decemlineata* to consume less leaf tissue and reduce the number of larvae found on treated plants (Aflitto & Thaler, 2020; Hermann & Thaler, 2014). Subsequent research has shown that plants that are pre-exposed to the predator semiochemical respond by becoming less palatable to *L. decemlineata*, illustrating a change in plant behavior (Aflitto et al., in prep).

Adult male *P. maculiventris*, releases sizeable amounts of an aggregation pheromone from a dorsal abdominal gland (DAG). At least two common GLVs are released from the DAG that plants also release when damaged. Although previous research has shown that the semiochemical elicits a response in the beetle prey, and changes in plant quality that negatively affect the prey, it is not clear if the plant is detecting and identifying the blend as information specifically from the predator. An alternative pathway for the change in plant behavior is that the semiochemical blend, which also shares greater than 60 percent of the same compounds as the potato GLV profile, is causing the plant to induce chemical defenses as if it were receiving a signal from a damaged plant, increasing defensive compounds, and reducing plant quality for the herbivore. For example, studies using *P. maculiventris* semiochemical treatments report using two compounds that overlap with potato plant GLVs that are also released from herbivore damaged potato leaf tissue: (*E*)-2-hexenal and linalool. Further, plant exposure to (*E*)-2-hexenal is known to prime trypsin protease inhibitors in a related plant species, *Nicotiana attenuata* (Kessler, Halitschke, Diezel, & Baldwin, 2006), which can be detrimental to feeding herbivores (Ryan, 1990; Zavala, Patankar, Gase, Hui, & Baldwin, 2004).

To understand more about the convergent use of the same cues, we investigated the effects of dispensing the synthetic GLV-containing DAG semiochemical of *P. maculiventris* and measured the behavioral response of *L. decemlineata* and *S. tuberosum*. By fractionating the DAG blend into the components that overlap with the prey's host plant and those exclusive to the predator, we were able to specifically ask two questions: 1) Does *L. decemlineata* reduce colonization, feeding, or oviposition in the full semiochemical blend and shared cues treated plots? 2) Is *S. tuberosum* responding to the GLV-containing shared cues treatment?

## METHODS AND MATERIALS

On May 24, 2019 *Solanum tuberosum* (cv Yukon Gold) seed potatoes were planted at the Cornell University's Homer C. Thompson Vegetable Research Farm in Freeville, NY using 0.3 meter interplant spacing, with 1 meter between rows. In total, two blocks of 13 rows were installed, separated by a 4-meter service road. The field was fertilized at the time of planting using triple 13 NPK at a rate of 733kg/ha.

Prior to the emergence of potatoes, experimental *L. decemlineata* were collected from wild populations in the surrounding farm by use of potted potato trap plants. The overwintering adults were collected from trap plants every 2-3 days and brought back to the lab until the study start. While waiting to be rereleased, *L. decemlineata* beetles were maintained on Yukon Gold potato plants under an 18:6 (L:D) light cycle.

The study was initiated on June 19, 2019. One hundred and eighty plots were installed consisting of a single plant marked by a pin flag. Three different treatments and a control plot were randomly installed throughout the site. The treatments included a full 5 compound blend mimicking *P. maculiventris* pheromone (full blend) (Aldrich et al., 1984), a fractionated shared cues blend which contained 2 compounds shared with the potato plant (shared cues), and a fractionated 3 compound blend which included compounds not shared with potato (predator only) (Table 1). Each dispenser was made from 2mL opaque brown Eppendorf tubes with 3 0.55mm holes in the top, acting as open dispersion dispensers. Each dispenser was filled with 600uL of one of the blends or remained empty in the control plots. The plot pin flag was used to hang the dispenser at an approx. height of 15cm. Fresh dispensers were installed every 2 weeks throughout the study period. Once the treatments were installed, a total of 600 adult beetles were rereleased to initiate colonization of the field site by introducing 50 beetles at evenly spaced intervals down each of the rows.

For each plot, we measured plant size, flowering time, tuber yield (end of season), the number of adults, egg clutches and number of eggs, number of larvae, and the amount of new plant damage by *L. decemlineata*. New plant damage was measured by using a focal leaf on each plot plant. The focal leaf was marked by a small piece of flagging on the 2nd or 3rd fully expanded leaf from the top. The focal leaf was moved to an undamaged leaf each site visit. This prevented double counting damage, avoided instances of missing herbivory where the leaf was fully consumed, and controlled for leaf age as the plants grew over the study. Damage was measured with a $mm^2$ grid printed on clear acetate on all site visits except for the last due to the high amount of damage. Instead, percent damage was estimated by 20% increments. In total, 5 site visits were completed between June 21 and July 19, 2019.

We tested the hypothesis that the treatment affected *L. decemlineata* behavior using mixed models. Hurdle models were used due to the large number of observed zero values in the response variables. A binomial generalized linear mixed model was used to model the probability of the presence or absence of each response variable (plant damage, adult colonization, oviposition, or larval abundance). In each model, treatment and plant size were included as fixed effects and plot and time of measurement were included as random effects. A linear mixed model was used to model the effect of the treatment on the non-zero amount of average plot damage and larval abundance. Plant trait measurements were analyzed using mixed effect models with plot as a random effect. Data points were transformed to meet the linear model assumptions of normality and homogeneous variance when appropriate. Note, the data presented in the figures are not transformed. Linear models were not fit to the oviposition and adult colonization data due to the low number of non-zero values. All analyses were completed using R statistical software (R Core Team 2014). Mixed models were analyzed using the lme4 package (Bates, Maechler, Bolker, & Walker, 2015)

## RESULTS

### Beetles

The shared cues treatment that contained the shared GLV compounds, and the full semiochemical blend treatment, which also contained the GLV compounds elicited the most consistent responses. Adult beetle presence was marginally reduced by the shared cues by 33 percent ($Z$ = -1.81, p = 0.071) and by 37 percent in the full blend ($Z$ = 2.02, p = 0.044). The presence of larvae was marginally reduced by the shared cues ($Z$= -1.87, p = 0.062). The average number of larvae per plot was reduced by the shared cues and predator only treatments, and marginally by the full blend (shared cues: T = -2.31, p

= 0.021, predator only: T = -2.20, p=0.028, full blend: T = -1.81, p = 0.071) (Fig 4.1). Oviposition was not affected by any of the treatments.

### Plant Damage

There was a 37% reduction in average plant damage in shared cues plots (*T* = -2.48, p = 0.014) and a 41% reduction in the full blend (*T* = -2.03, p = 0.044) (Fig. 4.2). On July 17, the last measurement day, the percent damage was assessed for all of the study plants rather than the mm consumed due to the high amount of damage, providing a season-long damage estimate. Both the shared cues (*T* = -2.48, p = 0.014) and full blend (*T* = -2.03, p = 0.044) exhibited lower damage levels (Fig. 4.2). Plant damage was not affected by the predator only treatment (*T* = -0.85, p = 0.398)

### Plant Measurements

By the second site visit (day 7 of the study) there was a clear difference in flowering in shared cues plots (Z = 2.35, p = 0.019) (Fig. 4.3). The shared cues plots also had larger plants for all measurement dates except for day 1 (d7 – *T* = 2.23, p = 0.027, d12 – *T* = 1.95, p = 0.053, d19 – *T* = 2.28, p = 0.024) (Fig. 4.3). At the end of the season, plant yield was not significantly different across the treatments (*T* = 1.33, p = 0.184) (Fig. 4.4).

## DISCUSSION

We found that the shared cues reduced herbivore feeding by 37 percent and the full blend reduced feeding by 41 percent compared to the control blend. Adult beetle presence was also marginally reduced by the shared cues by 33 percent and by 37 percent in the full blend. The predator only compounds did not display biological activity except for the average number of larvae found on a plot. Plants were also found to change their behavior in the shared cues plots by increasing flower initiation by 51 percent and growing slightly larger over the season. Tuber yield trended higher in the shared cues plots, however was not significant. These results along with previous research show that predators, prey, and plants in this tri-trophic system all respond to shared components of the predator pheromone.

Interestingly, the synthetic aggregation semiochemical of *P. maculiventris* caused changes in their prey's host plant. Although the presence of the full pheromone and the shared cues blend appeared to negatively affect the prey's behavior, this result could be due to an interaction between the predator odors and prey or between the host plant and herbivore or both. A previous study supports both being involved, where it was found that plants pre-exposed to the full pheromone and the present exposure of the full pheromone, where the prey is directly interacting with the compounds, both elicited reduced feeding and weight gain in larvae (Aflitto et al. 2021, in prep). Thus, in the present field study, it is probable that the prey was affected directly (via the detection of compounds) and indirectly through changes in the plant. This illustrates the multi-trophic potential of using shared cues to modify the behavior of organisms under management, such as in agricultural systems. Traditionally, semiochemical pest management acts on a single trophic level such as mating disruption, attraction to traps, or repelling insects from an area under protection.

The current semiochemicals on the market that contain shared cues may be inadvertently acting on multiple trophic levels. For example, benzaldehyde is commonly dispensed in orchards to attract plum curculio (*Conotrachelus nenuphar*) to traps (Piñero, Wright, & Prokopy, 2001; Prokopy, Chandler, Dynok, & Piñero, 2003). Benzaldehyde is also released by plants and is one of the most widely distributed floral VOCs (Farré-Armengol, Fernández-Martínez, Filella, Junker, & Peñuelas, 2020), with a known attractiveness to pollinators (Huber, Kaiser, Sauter, & Schiestl, 2005; Theis, 2006). Further, new active semiochemical release devices being used such as aerosol 'puffers' are capable of releasing significantly larger volumes of compounds when compared to more traditional passive open dispersion devices. The vastly higher release-rates raises the potential for plant tissue to come into contact with active compounds, and there is growing evidence of the effect on crop plants. Phytotoxic effects have been documented near aerosol release devices (Benelli, Lucchi, Thomson, & Ioriatti, 2019; Giroux & Miller, 2001), and although the mode of action has been tentatively attributed to nonspecific membrane disruption on the leaf surface, further research is needed to understand if, when shared cues are being dispensed, the plant is perceiving and responding to the treatment. In support of a more specific plant response to shared compounds are reports that semiochemical efficacy is increased when the surrounding plants are in regular contact with the compounds. Stelinski et al. reported that compounds released from aerosol devices can accumulate on nearby foliage and may result in higher concentrations of the semiochemical being present (Stelinski, Gut, Haas, McGhee, & Epstein, 2007) and the exposure of apple foliage to dispenser compounds has been shown to enhance pest control in some cases (Suckling, Daly, Chen, & Karg, 2007; Suckling, Karg, & Bradley, 1996). The explicit applied use of shared cues has yet to be widely considered and may hold novel treatments for plant protection in the future. Our study provides a proof of concept, where shared cues that are used by several trophic levels in a system reduces plant damage and increases plant growth. Additionally, the full semiochemical blend is a known aggregation pheromone for the beneficial generalist predator *P. maculiventris* which may recruit wild predators to a field (Fig. 4.5).

The shared use of volatile chemical cues between plants and insects has long been a point of interest among chemical ecologists (Rodriguez & Levin, 1976). Thomas Eisner in 1970, had a brief section in a book chapter dedicated to the remarkable overlap in secondary metabolites between plants and insects (Eisner, 1970), where he points out that "Some of the substances in arthropods, such as (*E*)-2-hexenal,…are in fact widely distributed among plants.". Whether we are artificially introducing sensory cues, such as with semiochemical treatments, or working to better understand ecological interactions, a greater knowledge of shared sensory cues will reveal the impact and interconnectedness of certain cues in systems.

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Table 4.1 Percent and volume of compounds contained in each volatile field dispenser treatment blend. Control dispensers were empty and did not contain any compounds.

| Blend | *(E)-2-hexenal* | *alpha terpineol* | *benzyl alcohol* | *linalool* | *terpinen-4-ol* |
|---|---|---|---|---|---|
| *Shared Cues* | 99.33%<br>(595.98uL) | - | - | 0.67%<br>(4.02uL) | - |
| *Pred. Only* | - | 90.85%<br>(545.1uL) | 8.30%<br>(49.8uL) | - | 0.85%<br>(5.1uL) |
| *Full Blend* | 59.58%<br>(357.48uL) | 36.36%<br>(218.16uL) | 3.32%<br>(19.92uL) | 0.40%<br>(2.4uL) | 0.34%<br>(2.04uL) |
| *Control* | - | - | - | - | - |

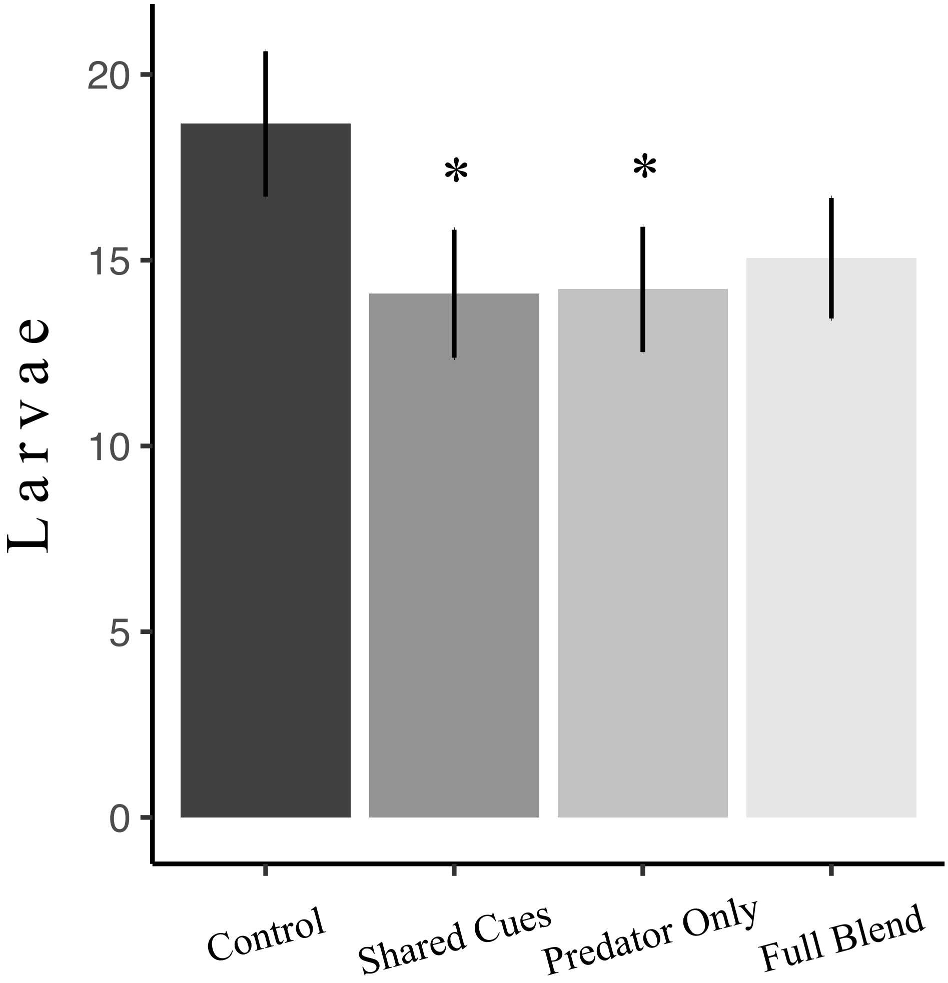

Figure 4.1 Mean number of *L. decemlineata* larvae found on *S. tuberosum* field plots over the study period. Shared Cues represent the compounds that are shared with potato plants, Predator Only represent the compounds only contained in the *P. maculiventris* aggregation pheromone, and the Full Blend represents the full *P. maculiventris* aggregation pheromone. Bars represent the standard error of the mean. Asterisks denote a p-value < 0.05.

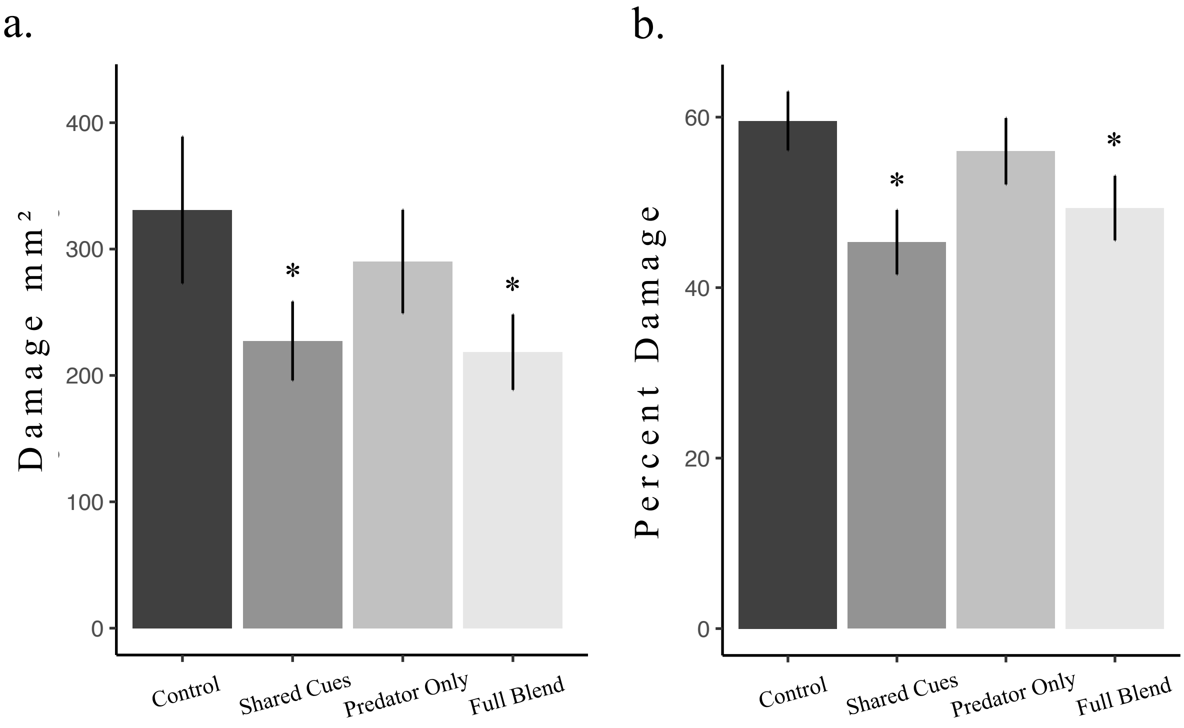

Figure 4.2 (a.) shows the average mm$^2$ over the measurement period in *S. tuberosum* field plots where the shared cues and full blend had lower damage. (b.) shows the end of season total percent damage estimate (estimates based on 20 percent increments), which followed the same damage trend as the average mm$^2$. Shared Cues represent the compounds that are shared with potato plants, Predator Only represent the compounds only contained in the *P. maculiventris* aggregation pheromone, and the Full Blend represents the full *P. maculiventris* aggregation pheromone. Bars represent the standard error of the mean. Asterisks denote a p-value $< 0.05$.

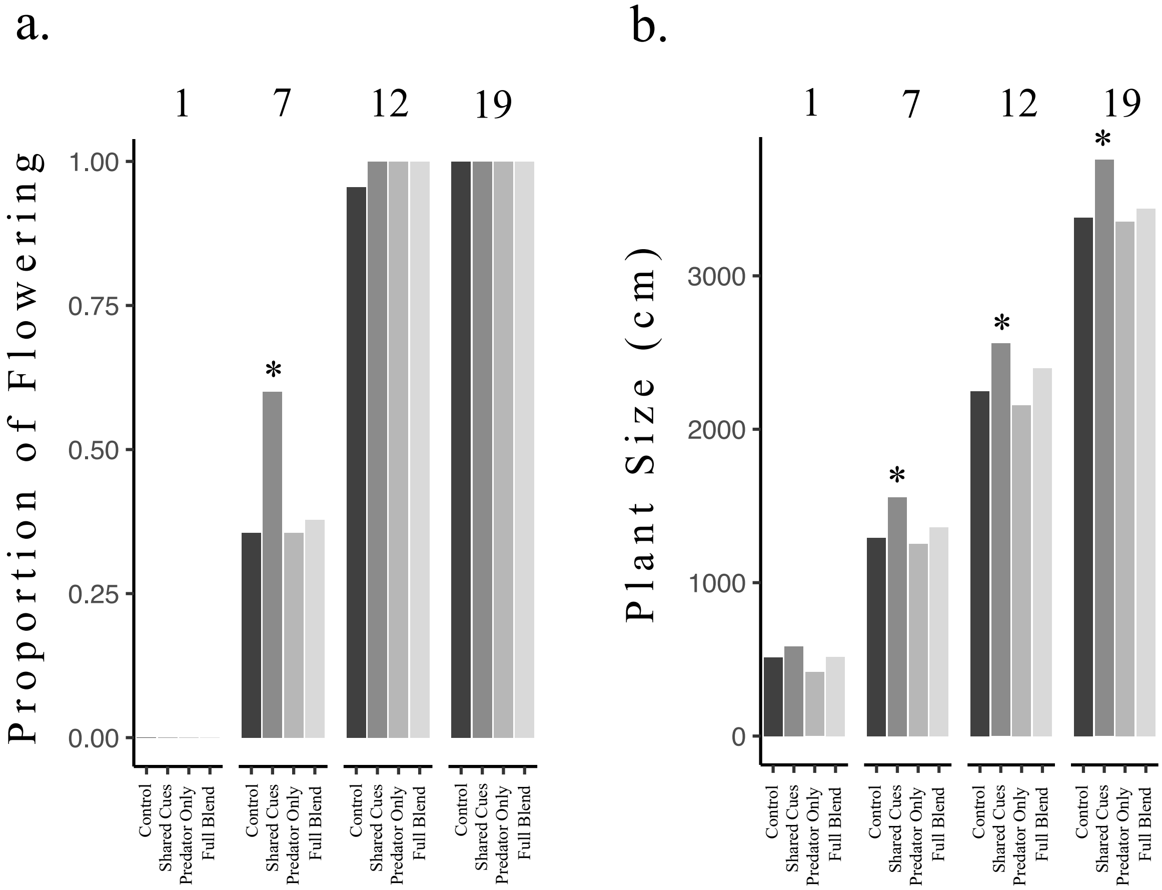

Figure 4.3 Each block represents the days since the field study start (i.e. 1, 7, 12, 19). (a.) shows a pulse of *S. tuberosum* flowering on day 7 in the shared cues treatment and a slight increase in the full blend. (b.) shows the shared cues consistently had larger plants over the study period. Plant size in the full blend trended non-significantly larger. Note volatile treatments were placed 3 days prior to the first measurement date. Shared Cues represent the compounds that are shared with potato plants, Predator Only represent the compounds only contained in the *P. maculiventris* aggregation pheromone, and the Full Blend represents the full *P. maculiventris* aggregation pheromone. Asterisks denote a p-value < 0.05.

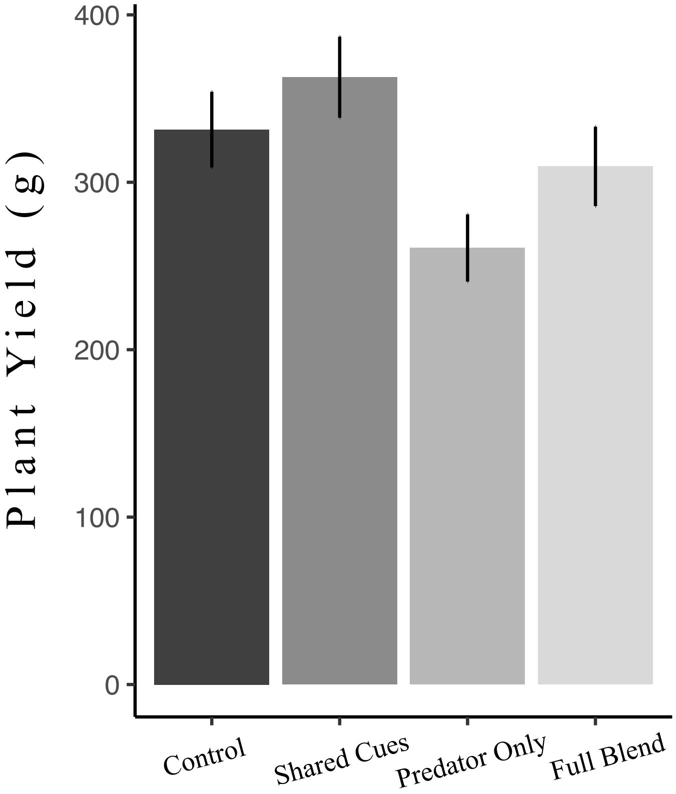

Figure 4.4 Mean tuber yield per plant in grams. Shared Cues represent the compounds that are shared with potato plants, Predator Only represent the compounds only contained in the *P. maculiventris* aggregation pheromone, and the Full Blend represents the full *P. maculiventris* aggregation pheromone. Bars represent the standard error of the mean.

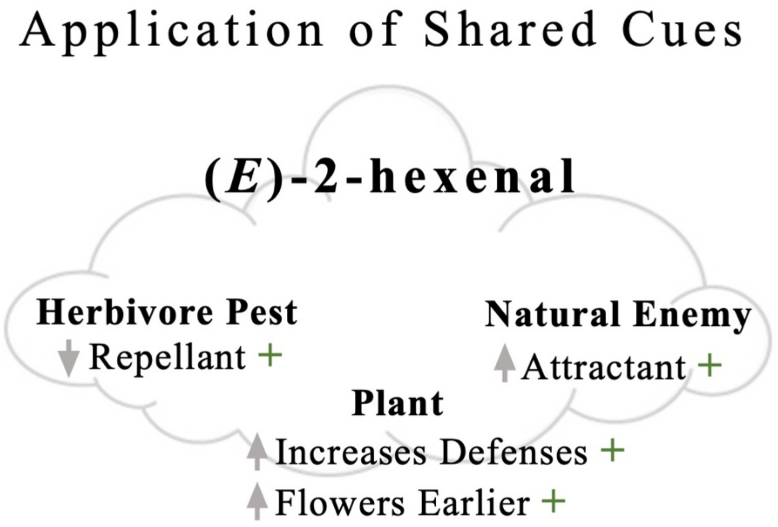

Figure 4.5 Conceptualization of applying shared cues to meet insect management objectives. Positive signs indicate the net result to the plant. Since all levels in the tri-tropic system detect and use (*E*)-2-hexenal, there is the potential to modify the behavior of multiple organisms, resulting in the greatest benefit to the plant. This is contrasted by the current use of single, target organism-based semiochemical treatments. It is important to point out that the specific implications of a certain compound will vary from system to system and may not always yield a net positive benefit.

## Appendix

# How Search Engine Optimization Can Increase Extension Content Visibility and Strengthen Impact Statements

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## Abstract

The near ubiquitous use of search engines has provided new forms of data, such as what people are searching for, the locations of their searches, and what content they prefer. In this paper, we highlight the value of this data for extension professionals and others in academia to inform programs and content that meet the specific needs of their clients. We argue that a broader adoption of these concepts will help modernize cooperative extension programs in the digital age and in turn allow us to better serve the public.

Keywords

search engine optimization (SEO); search data; information technology transfer; impact statement; SEO for extension content

## Introduction

How people find and consume information has changed a great deal since the Smith-Lever Act was signed into law in 1914, creating a national Cooperative

Extension network we know today (§e372, 1914). At that time, telephones were not yet a common household fixture and homing pigeons were still used to send important wartime messages. It goes without saying that the ways in which clients interact with extension content have vastly changed with the onset of digital technology, search engines, smart phones, and social networks. How people find, consume, and share information has completely transformed how organizations determine what content to produce, promote, and measure impact. Seventy-one percent of people turn to search engines to research new products, brands, or services, positioning search engines as a critical touch point for the public to access information they need (Forrester, 2016). The significance of this search behavior forces us to ask if extension is producing, optimizing, and evaluating content to meet the needs of modern clients, or is our approach more akin to the humble homing pigeon? In this paper we outline three primary areas where cooperative extension has opportunities to capitalize on this digital behavioral shift to create more awareness and exposure for Extension programs and services.

First, we introduce basic search engine optimization (SEO) principles which are a critical part of how online extension content is found by the general public. Next, we discuss how the use of digital marketing tools can illuminate content opportunities that respond to areas of high public search interest. Finally, we propose a novel way to use digital data to evaluate programs and bolster impact statements using client web behavior.

## Increasing the Reach & Relevancy of Extension with Search Engine Optimization

Optimizing content for search engines provides extension with an opportunity to serve the public's needs at the right place, at the right time. Just like an ecosystem is comprised of interdependent living and nonliving components, the digital landscape can be thought of in much the same way. When we simply create digital content (nonliving), a critical part of the ecosystem is left out - how people find and interact with it (living).

Consider your own behavior when searching for information online. If you are like most people, your quest for information starts with a Google query (Percell 2011; Zickuhr & Madden 2013). Results that appear on the first page of Google's search results page (SERPs) receive 92% of all traffic from the average search, with 33% of traffic going to the top-ranking search result (Chitika 2013). Moreover, 72% of participants in a Forrester study reported that search engine results are trustworthy (Forrester, 2016), indicating search results serve as a trust building first touch point for relationship building. This search behavior significantly increases the awareness and reach of the top-ranking websites, allowing site owners to drive large volumes of organic search traffic into their content. Driving engaged traffic to extension websites via organic search results is an opportunity to efficiently meet the public's search interests and to foster the relationship between extension services and the public. As more people visit extension websites to consume content, we increase the likelihood of future interactions - both online and offline. The search results may be the first time someone becomes aware of the existence and value of extension services although the impact of this first introduction has a long lifetime value, including future off-line interactions such as educational programs, events, and services that may serve someone’s needs for years to come. Search serves as a launch pad for a long-term trusting relationship between the public and Extension.

Imagine someone traveling into Manhattan who turns to Google after hearing someone in the elevator say that their hotel has bed bugs. They are urgently seeking information to identify bed bugs, which will help them decide whether or not they should switch hotels. This is an opportunity for extension content to reach this person at exactly the right time. The traveler gets to an extension bed bug fact sheet which helps them identify that, indeed, their room has bed bugs. The traveler switches hotels and avoids not only bites, but also spreading bed bugs back to their home. By leveraging content in the search results, extension served an important role in helping someone make a decision that significantly impacts their quality of life. Now, let’s fast forward a few years and this same person discovers shiny green beetles around their garden, and as they become concerned, they recall their experience with extension’s bed bug content. Now that they are aware of and trust extension content, they know exactly where to turn. They go into the extension website, identify their garden pest, and sign up for a Master Gardeners workshop where they can learn more about garden pest prevention. This is an instance in which the extension built relevancy and trust using the power of search engines, which later translated into off-line engagement with an organization this person would otherwise know nothing about. Extension is in a unique position by not relying on advertising or product sales to drive revenue. Due to this fact, users may be more likely to trust extension content without being sold on a specific product or service. This puts extension in the position of being a trusted authority, wanting nothing more than to serve public needs.

This is why SEO is so important and precisely the reason private industry invests time and money optimizing their content, regularly outcompeting extension sites for coveted top search results. Perhaps Jimmy Wales, the founder of Wikipedia, says it best, “If it isn’t on Google, it does not exist.” There is currently a missed opportunity for extension to reach a larger audience, and relatively small changes can elevate extension content in search results.

## How Does SEO Work?

The major search engines use over 200 different factors that help determine the most relevant content to display in search results (Search Engine Land 2017). While no one knows all of these factors, we do know at a rudimentary level that search engines evaluate sites on three major factors: Content, Relevancy, and Architecture. Search engines want to know whether or not your site is producing high quality content and whether you are using the right keywords on your page. Secondly, search engines look for relevancy, which they measure by how many other websites are linking to your domain. This includes links shared in social media platforms which send strong signals to Google about the quality of your content. This concept of other people placing links back to your content is roughly analogous to citations in academia. We will discuss this more below. Thirdly, sites are evaluated from a technical architecture perspective - can search engines crawl all pages on your site, can they read the content, and are the important pages about your site prominent?

When content is posted online, search engines use bots to “crawl” this content to gather and index the information it contains (Brin and Page 1998). In the most basic sense, SEO is essentially the practice of making the bot’s job easier by telling it clearly what content your site has to offer. Keywords are one of the primary tools a bot will use to organize content. Several locations are scanned for keywords including the meta data located within meta descriptions, page titles, navigations, anchor texts, image alt texts and file names, and instances of a specific keyword or a semantically related keyword used several times within the body copy. Generally speaking, keywords should always be integrated in a natural, unforced way, avoiding overuse or “keyword stuffing” for which search engines will penalize sites.

Another important input for search algorithms is how many high-quality inbound links point back to a domain. This is referred to as domain authority. Domain authority operates on a scale of 1-100, and every website has a score (MOZ 2018). If a website has a significant volume of high-quality inbound links from other high domain authority sites, it drives up the domain authority and sends signals to search engines to rank that website’s content higher. Inbound links from low quality sites will not drive-up domain authority. Domain authority is an assessment of credibility or trustworthiness of a site. If a lot of credible websites link to your site, it is an indicator that your site is trustworthy, and thus Google favors your site in the SERPs. If no other sites link to you (or spammy sites), it may be an indicator that your content is not very high quality, and therefore Google will not rank your site's content very high.

While there are more factors that contribute to domain authority, the important part here is that extension sites often have high domain authority compared to other competing content sources since they are commonly hosted with the Land Grant University's root domain. For example, in New York State, Cornell Cooperative Extension sits on a subdomain within the cornell.edu domain, which has a domain authority of 95 out of 100. With intrinsically high domain authorities, extension has an opportunity to outrank commercial counterparts for important keywords that can drive high volumes of organic search traffic, and therefore reach a broader audience.

It is worth noting that some of the top shared extension related content stem from enlisting the help of local media to spread information. This finding is significant with regards to domain authority in that extension services producing high quality content may also be able to rely on local media to help expand the reach of their content in local communities while simultaneously building valuable inbound links.

## How Extension Can Leverage Search Data

For the first time in history, we can see specifically what information clients are seeking by utilizing certain tools that provide search data and content sharing analyses. In a way, this shifts how extension interacts with clients from a primarily top-down relationship, where research is disseminated to clients, to bottom-up, where clients can drive what extension produces. Clearly both are important to meet mission goals, however the bottom-up method has been largely limited to tools like surveys (Patton and Kaminski 2010), which may yield biased results if they are only distributed to email lists of existing users. Using search data allows us to see a broader view of what topics have the highest search demand within a specific country, state, or county. This knowledge allows us to create content that matches these areas of high search interest. In marketing, this is often referred to as "inbound marketing" or "pull marketing" which focuses on attracting customers by responding to what they are looking for by creating content that reflects areas of high search demand.

### The Power of Keyword Research

Keyword research is an important process that can uncover areas of search interest, a methodology rarely used in Extension. There are several powerful tools commonly used by digital marketers to do this: Google Keyword Planner, SEO MOZ Keyword Explorer, and SEMRush. These tools provide approximate monthly search volume (MSV), seasonal search trends, and suggestions for often overlooked relevant keywords. While none of these tools are perfect, they can, in combination with tools like Google Trends, Buzz Sumo, and Google Analytics, help provide an understanding of what search topics and specific keywords people are using.

## Case Study

Consider a rapidly expanding invasive species, the brown marmorated stink bug. Most state Extension Services in the current range of this pest have content on the crop damage it can cause. Since this bug has a wide host range that overlaps with many of the plants we like, it is no surprise there is an abundance of agricultural information on this bug. However, looking deeper into what clients are searching for reveals a part of the story that is missing. In the interest of brevity, we will again narrow our focus on New York State.

In our stink bug example, Google Keyword Planner shows that interest in the brown marmorated stink bug is quite high in New York State. Having an approximate monthly search volume (MSV) of 18,100 makes this insect the second most searched pest in New York. A helpful feature of Google Keyword Planner is the Keyword Ideas associated with your initial inquiry. In this case, the Keyword Ideas show that fewer people are looking for information about the damage these pests can inflict on plants, but rather there is more interest in information about human-bug interactions.

Indeed, the top related keywords include inquiries like “Do stink bugs bite?”, “stink bugs in house”, and “What do stink bugs smell like?” Not only does keyword research highlight potential Extension content gaps, it reveals opportunities to increase Extension’s reach to a broader audience. By creating content that addresses the question, “What do stink bugs smell like?” extension can drive search traffic within a new audience segment. A little research shows that when disturbed, the bugs release decenal, a compound that gives cilantro its smell. Consider all of the creative content extension could produce to address this search interest while at the same time raising awareness about this invasive species and broadening cooperative extension's reach and long-term value in the process.

With the powerful combination of quality content, SEO implementation, and high domain authority, extension has an enormous opportunity to meet public search interest while expanding reach and relevancy with a new generation of users.

## Using Search Volume Insights to Aid Program Evaluation

The third way that extension or even grant writers may benefit from the use of search data is in program evaluation - both on the front end of program or grant selection and the backend of performance evaluation.

On the front end, nearly every topic and program could gain learnings from search interest insights. Keyword research helps inform both content creation and the tactical application of keywords to the website's content. In many ways, keyword research allows a grant writer or a content creator to take the guess work out of topic selection by providing assurances that the resources spent will meet public demand. This methodology helps teams evaluate potential impact, prioritize content production resources, and anticipate returns on investment. By understanding search volumes, Extension can better predict where to hedge its bets to respond to the public's areas of high search interest and where to justify grant spending.

On the back end, once a program has launched, extension can use tools that crawl newly built inbound links and social sharing statistics to evaluate the reach and engagement of its content. Tools like SEO Moz, or Buzz Sumo show inbound links built to any webpage. As discussed above, if a piece of content is particularly successful, it is likely to be widely shared on social media and produce quality inbound links from other credible websites. Much like citations in academia, if a content piece is widely linked to and shared on social media, it is a strong indicator of high reach and impact.

Another commonly used tool to evaluate impact of a specific program is Google Trends. Google Trends shows upticks or declines in search interest over a specific time frame. While relatively simple, this is an important way to measure program interest. Theoretically, if a program or content piece had a wide reach and engagement, Google Trends would reflect this rise in interest. In the private sector this is commonly used to evaluate the impact of advertising or awareness for a brand or product. Similarly, Extension may be able to utilize Google Trends to identify how impactful a project was in spurring search interest then use these insights to bolster impact statements. The list below outlines the general process from identifying content topics through to measuring impact (Table 1).

Table 1

A generalized 8 step process using search volume insights starting with content ideation through to program evaluation and promotion:

1. Identify Areas of Search Interest - Google Trends and Keyword Planners are used to identify topics of high search demand, keyword opportunities, and seasonal interest patterns.
2. Analyze Content Trends - Buzz Sumo is used to determine what content already exists on a topic, what content formats are popular for their target audience, and what whitespace exists for new content.
3. Evaluate Opportunity - Evaluate potential impact of a program and allocate proper resources to content creation and promotion.
4. Create and SEO Quality Content - Create content then apply SEO best practices to webpages.
5. Promote Content - Promote content on social media, on relevant publications, and enlist local media to help increase awareness of topic driving inbound links and public interest into content.
6. Drive Deep Engagement - Use content as an opportunity to encourage people to engage more deeply with services by getting them to take meaningful actions such as signing up for an email newsletter, or an upcoming workshop.
7. Measure Impact - Measure the success of a program by assessing the webpage's organic search traffic (typically via Google Analytics), inbound links, Google Trends search interest spikes, and social sharing metrics.
8. Use Data to Create Feedback Loop - Utilize the performance data to modify the content or apply learnings to future content opportunities. An ongoing feedback loop helps optimize existing content and strengthen future development.

## Conclusion

SEO is a powerful tool that can help extension build awareness and engagement in the digital age. Internally, utilization of search and performance data analysis will allow extension agencies to better predict what content to produce and to evaluate program performance. While this methodology is widely used in the private sector, it is relatively untapped in the extension realm. Part of this under utility may be due to several practical reasons - including lack of skill development opportunities for extension employees to learn SEO, limited time and resources to perform extra job duties, and technical content creation constraints created by a website's content management template. While these are all legitimate reasons for low adoption, it would behoove extension agencies to offer ample digital education and training opportunities to employees as well as invitations to discuss SEO needs with other stakeholders such as program marketing directors, website developers, and SEO consultants. Though SEO does take some time to gain traction, once a formalized process is initiated and initial learning curves are over, it will be a great benefit to all parties to implement search insights and learnings into extension programs. On the back end of program evaluation, utilizing data such as inbound links and social sharing data will help researchers evaluate the impact of their work.

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