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Chemical-Mediated Mate Attraction in Clover Root Curculio (*Sitona hispidulus* F.), A Belowground Pest of Forage Crops

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CHEMICAL-MEDIATED MATE ATTRACTION IN CLOVER ROOT CURCULIO (SITONA HISPIDULUS F.), A BELOWGROUND PEST OF FORAGE CROPS

by

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Abstract

Curculionid beetles are well known for their production of semiochemicals, including contact (e.g., cuticular hydrocarbons) and volatile pheromones that mediate conspecific attraction and mating. Clover root curculio (CRC; *Sitona hispidulus*) in particular is an emergent, soil-borne pest of significant concern to producers of alfalfa and other leguminous crops in the United States. Producers have few control options for CRC and a greater understanding of chemical-mediated attraction in this species can potentially improve the sustainability of its management. To examine which chemical cues are important for affecting the behavior of CRC and whether there are sex-based differences in response, I performed behavioral assays using an olfactometer (i.e., Y-tube). This effort was complemented with solvent-based extractions of cuticular hydrocarbons and analysis via Gas Chromatograph Mass Spectrometer (GC-MS) to characterize contact chemicals that can also contribute to conspecific recognition and attraction. Olfactometer assays suggest that CRC most likely has a male-produced, volatile aggregation pheromone, as females exhibited a strong preference for male odors. In contrast, our cuticular hydrocarbon data suggest a lack of sex differences in their epicuticular wax layer. Knowledge of chemicas involved in mediation of Curculionidae mating interactions is essential for the development of ecologically sound strategies for the control of below-ground pests. Once semiochemicals are identified and behaviors observed, we can use this knowledge to develop a cost-effective detection method, such as semiochemical-baited traps. These traps could alert growers of the presence of CRC in their fields, disrupt mating, as well as draw some away from their crops.

Acknowledgments

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I would also like to thank the other people who helped me with this project. Michelle Grilley for being with me through the ups and downs in the field and data collection. The person who figured out the wires were connected wrong in the electroantennogram circuit and fixed it. I would like to thank Calvin Luu and Kim Hageman's Lab for their help with the cuticular hydrocarbon analysis. Calvin showed me how to use all the instruments and how to prepare the samples. Ricardo Ramirez and his lab conducted a lot of preliminary data and provided us with the vacuum and sieves. The intel and connections that Ricarob had were invaluable for finding the alfalfa fields, coordinating with growers and connecting to the community.

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INTRODUCTION

Belowground pests are a cause of significant concern for growers. Root feeding by insects during their soil-dwelling life stage, including those from the orders Coleoptera, Diptera, Homoptera, Hymenoptera, Lepidoptera, and Orthoptera (Brown & Gange, 1990), can often cause large monetary losses. These species decrease host establishment, disrupt nutrient and water uptake, and facilitate exposure to soil-borne secondary pathogens (compounding losses) (Hunter, 2001). Pests that attack crop roots remain among the most economically damaging and challenging to control in agroecosystems.

Advances in chemistry allow for a greater exploration of compounds important for insect attraction and mating, encouraging integration into pest control methods such as traps or baits. Indeed, recent reviews have highlighted that approximately 75 different chemical compounds can elicit behavioral responses among root-feeding insects, with the large majority having attractive properties (Landolt, 1997). Such sensitivity is critical during their soil-dwelling life stage, as root-feeding herbivores use tactile and chemical-mediated cues to locate resources in the soil matrix, being unable to rely upon visual stimuli (Hiltpold & Turlings, 2012). Other semiochemicals (external chemical signals), such as pheromones, which facilitate communication between the same species, or allelochemicals, which interact between different species, can also aid in pest monitoring and management. Their use also often has many benefits, including being host-specific and having bioactivity at low concentrations (Witzgall et al., 2010).

Curculionid beetles (Coleoptera) are well known for their production of aggregation pheromones (Blight & Wadhams, 1987), in addition to their potential ability to sense microbial cues which can inform host-plant selection and egg laying (Johnson et al., 2005; Wolfson, 1987). For example, larvae of the clover root weevil (*Sitona lepidus*) have been observed to be attracted to the isoflavonoid formononetin, a compound produced in high concentrations in root nodules of white clover, *Trifolium repens* (Johnson et al., 2005). In addition to larvae, maternal clover root weevils living aboveground also seem to be able to detect these cues, because they preferentially oviposit on *T. repens* with abundant root nodules (Johnson et al., 2006), which provides a strong advantage for offspring, whose survival and development is dependent on finding the nodule for feeding.

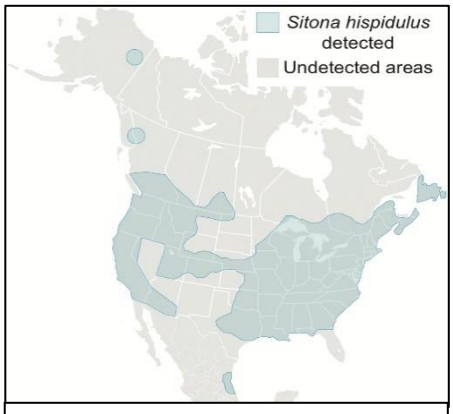

Figure 1. *Sitona hispidulus* throughout North America. Map adapted from Rim et al. (2019), created from distribution reports from Bright (1994) and Bright and Bouchard (2008).

Here in the Intermountain West, clover root curculio (CRC; *Sitona hispidulus*) has recently emerged as a significant below-ground pest of concern for alfalfa producers. Clover root curculio was originally introduced to North America in the late 19th century (Wildermuth, 1910) and now is widely distributed across the U.S. (Bright, 1994; Donald & Bouchard, 2008; Rim et al., 2019) (Figure 1). This expansion is likely tied to the historical shift from forage cropping systems with short-term clover stands to larger

acreage alfalfa stands where CRC persists for multiple years (Wildermuth, 1910). The recent phase-out of soil active broad-spectrum insecticides and synthetic soil fumigants (i.e., carbofuran and methyl bromide) in the early 2000s by the Environmental Protection Agency has also left producers with little to no options for control of soil-dwelling pests, and as a consequence, has likely contributed to CRC population growth and spread (Rim et al., 2019).

Alfalfa producers have few options for effective control of CRC. Currently, no commercial cultivars with resistance to CRC are available (National Alfalfa and Forage Alliance 2021), and diagnosing CRC-related damage in alfalfa stands remains a critical challenge. Often, damage caused by CRC is misdiagnosed as a nutrient deficiency or disease (Tietz, 2012; Wenninger and Shewmaker, 2014) or overlooked entirely because of the presence of more obvious aboveground pests. Furthermore, the cryptic nature of CRC eggs and larvae in soil, in addition to adult movement between the soil surface and plant canopy (Figure 2), makes CRC monitoring complex and labor-intensive for producers and farm advisors alike (Rim et al., 2019). Finally, biological control measures, including use of parasitoids, as well as entomopathogenic bacteria, fungi, and nematodes have been examined for their potential to suppress CRC with mixed success (Rim et al., 2019).

There is great interest in leveraging semiochemicals for pest management, both for monitoring and disruption of behaviors harmful to commodities (Howse et al., 1998; Witzgall et al., 2010). Given their oft species-specific nature and bioactivity at low concentrations, semiochemicals such as pheromones can be a powerful tool for pest monitoring (Witzgall et al., 2010). However, the exact compounds mediating CRC aggregation and host selective behaviors remain unknown. Their discovery, development, and introduction within an alfalfa integrated pest management (IPM) system could vastly improve management decisions, as well as reduce CRC populations through interference of feeding and host-selective behaviors.

CRC does not respond to the *S. lineatus* pheromone when field-tested in environments where both species are known to co-occur (Quinn et al., 1999; Reddy et al., 2018; St Onge et al., 2018; Toshova et al., 2009; Tóth et al., 1998). The discovery of a semiochemical from CRC would be an important step toward development of an effective trapping system.

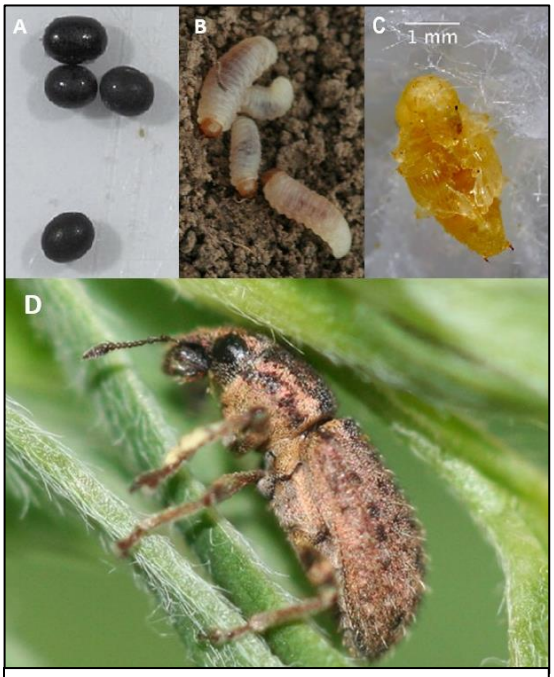

Figure 2. (A) Clover root curculio eggs (B) Larvae (C) Pupa (D) Adult on alfalfa host *Medicago sativa.* Charcteristic features include semi-erect hairs and short snout. Images adapted from Rim et al., 2019.

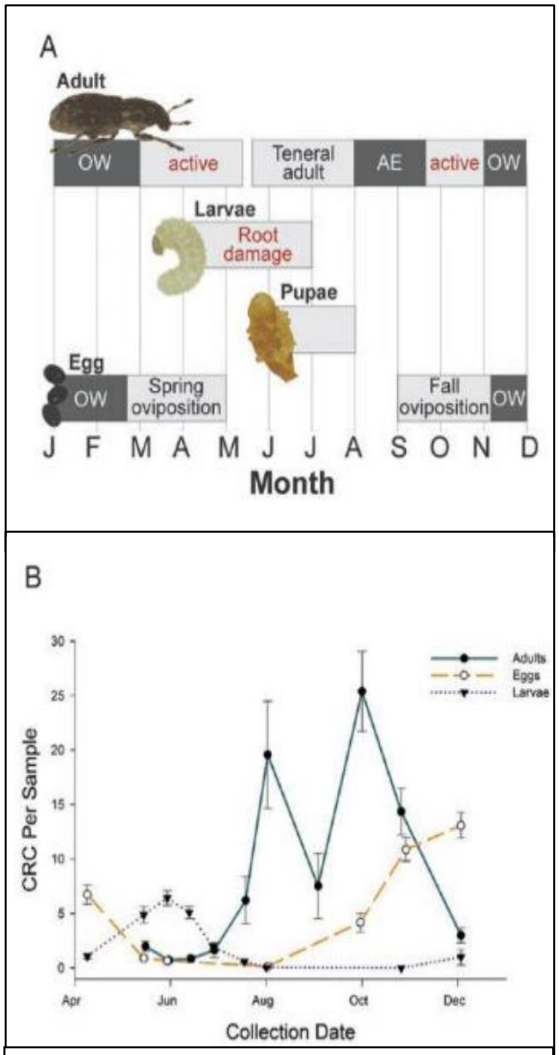

Figure 3. (A) Generalized life cycle of *Sitona hispidulus* throughout the United States. Distribution and life stage abundance may change depending on region. For example, in northern Utah, Price (2017) reported higher adult overwintering and thus lower springoviposition. AE: aestivate, OW: overwinter. (B) *S. hispidulus* adults, eggs, and larvae were measured through 4 D-vac samples, 8 egg samples and 8 soil cores from each field. Samples from 7 fields are averaged. Adult activity peaks in late August and early October.

To explore the chemical cues influencing CRC behavior, I conducted behavioral assays using an olfactometer, alongside solvent-based extractions of cuticular hydrocarbons for GC-MS analysis. These efforts shed light on both volatile and contact chemicals contributing to conspecific recognition and attraction. Understanding the chemical signals driving Curculionidae mating behaviors is critical for developing environmentally friendly and cost-effective approaches to below-ground pest management. Upon identification of semiochemicals and observation of associated behaviors, we can leverage this knowledge for detection methods, like semiochemical-baited traps. These traps have the potential to serve as early warning systems for growers, interrupt mating activities, and even redirect some pests away from valuable crops, offering a multifaceted solution to pest control.

METHODS

Study System

Generally, clover species (*Trifolium* spp.) were the preferred preferred host of CRC over alfalfa (*Medicago sativa*) and related legumes, however. However since the U.S. increased increased alfalfa production, CRC is increasingly more common. Alfalfa is now a foundational element of the agricultural economy of the Intermountain West. Its main uses are as hay forage for animals, given its high nutritional value, and as an important cover crop, improving soil health through additions of organic matter and nitrogen.

CRC exhibits the highest activity in the fall during adult emergence in the Intermountain West. (Figure 3). While soil and larvae are cryptic and hard to study, adults have been observed in early fall to continue feeding and start laying eggs after atthe aestivation (hibernation) period. There are discrepancies in the phenology between previous research on the East Coast and recent research in the Intermountain West. In eastern states, both eggs and adults overwinter and adults promptly resume oviposition in the spring (Bigger, 1930; Herron, 1953; Lau & Filmer, 1959). However, in

the western United States (e.g., Utah), recent research has indicated that CRC overwinters primarily as eggs and few adults survive to oviposit the following spring (Price, 2017). Mating behavior during the spring in the Intermountain West is still unclear but believed to have low activity.

Field Collection.

We can utilize above-ground activity for monitoring and trapping during fall migration and mating (Culik & Weaver, 1994; Leibee et al., 1981; Pausch et al., 1981). In a comparable system (*Sitona lineatus*), primary migration for more than 90% of adults is through crawling (Hamon et al., 1987). Clover root curculio distribution and abundance is likely highly variable and aggregated, based on previous observations. In 2020, members of our group sampled 15 fields in Northern UT with a suction sampling device (James et al. 1980, Roberts et al. 1982, Goldson and French 1983), confirming a clumped distribution pattern.

We visited alfalfa fields that were part of a wider pest monitoring effort for the state, administered by researchers at Utah State. We collected CRC by walking transects along the perimeter of each field. We used a modified hand-held vacuum (Husqvarna) to suction insects and some topsoil around the base of the alfalfa plants sampled. Insects were sorted on-site using sieves with descending size variations to separate them. Most curculio were found past the 5 mm sieve. CRCs were identified by their physical characteristics. Typically adults of 3-5 mm size have a short, broad snout and have semi-erect hairs on their wing covers (see Figure 3).

Sexing

Clover root curculio colonies are difficult to sustain in the laboratory (Wolfson, 1987); thus, adult CRC were collected and sorted in the lab. Recovered adults were placed in bug dorms (BioQuip Products Inc., Compton, California, USA) with a moistened cotton roll (Patterson Companies, Saint Paul, MN) and alfalfa bouquets, replenished every 3-4 days. We separated the sexes into smaller bug boxes to prevent further mating. Males and females were distinguished by their physical morphology. Males were identified by the distal abdominal segment (pygidium) overlapping the hypopleurites which causes dorsal exposure beyond the apex of the elytra. Additionally, the last sternite (abdominal segment) is straight (Leibee et al., 1981). Females are typically larger than males and the ventral edge of the last sternite is rounded (Bright, 1994).

Y-tube Olfactometer.

Male and female CRC behavioral responses to the opposite sex were tested in a two-choice, Y-tube olfactometer (ARS Inc., Gainesville, FL; Figure 4) using charcoal-filtered air at 0.8 L/min. The two choices that were placed at the end of each arm were a cutting of fresh alfalfa (control) or bulked batches of adults of the opposite sex (n=10), along with a cutting. Alfalfa cuttings were of approximate equal mass in each test performed. All assays were conducted with no illumination other than red light, at 25°C, and with 75% relative humidity. Groups of field-collected CRC weevils were separated into separate containers and transported to the bioassay room 1 h before

the tests. The stimuli at each arm of the olfactometer were connected to the air flow and left to equilibrate for 1 min before each test. In each test, we placed a single weevil at the entrance of the main olfactometer tube, and its response to the two stimuli was observed for a duration of 5 min. We recorded weevil behavior as follows: choice for male/female or control (i.e., weevils observed visiting a respective arm one or several times), no choice (i.e., weevils move but do not reach any arm, or visit both equally), and non–responding (i.e., weevils are inactive and do not leave the entrance of the main olfactometer tube). After each test, the weevil was discarded and the olfactometer cleaned and reset. The apparatus was cleaned with acetone and sat until acetone was completely airdried. The arm position of the stimulus was alternated between successive weevils to control for positional effects. The effect of each stimulus (opposite sex) was tested against control with 26 weevils/sex (n=52). This count does not include non-responding weevils, as they did not provide behavioral results. No choice responses were not included in first choice data analysis, but were included for average time spent.

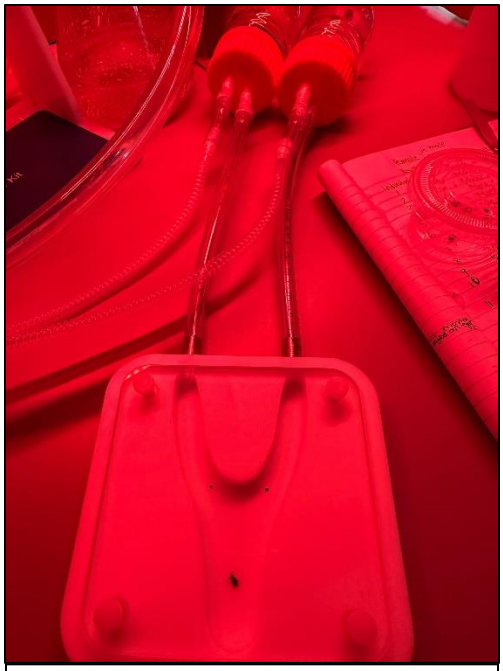

Figure 4. The olfactometer used charcoal-filtered air through bulked sex samples' head space. Tests performed in silence and under red light, which the weevils cannot detect.

Cuticular Hydrocarbons

We adapted our hydrocarbon extraction and GC analysis from Souza et al. (2021). Based on the previously discussed morphological assessment, males and females were sorted into glass vials (1.8 mL, Thermo Fisher Scientifc Inc.). We established three replicates of male and female bulked samples (five weevils per vial). Insects were added to 300 µL of hexane (Optima Grade, Fisher Scientific, New Jersey USA) and allowed to rest for 4 min, then agitated on a vortex mixer for one minute. Extracts were then transferred into new glass vials and stored at -20 °C until analysis.

Samples were dried under nitrogen at 35˚C. Initially, nitrogen was blown at 4.8 L/min for 3 min, then ramped up to 5.5 L/min for 3 min, followed by 6.0 L/min until completion. The samples were then resuspended in hexane (10 µL). Cuticular hydrocarbon composition profiles were obtained by analyzing the samples using a gas chromatograph (GC) (Trace 1310 Thermo Scientific) coupled to a mass spectrometer (MS) (TSQ 8000 EVO Triple Quadrupole) fitted with a silica capillary column (Restek Dtx-5ms, 15 mm x 0.25mm ID x 0.25 µm). Data was acquired under the following GC conditions—inlet temperature: 250 °C, carrier gas: helium at 51 cm/s, split ratio 13:1, transferline temperature: 280 °C, initial temperature: 40 °C, initial time: 2 min, rate: 10 °C/min, final temperature: 260 °C, for 6 min. The MS was held at 230 °C in the ion source with a

mass range of 35–500 mu and a scan rate of 4.45 scans/s. Chromatographs were viewed using Chromeleon v. 7.3.2 (ThermoFisher Scientific).

Data Analysis.

All analyses for this project were performed using R v.4.2.2 (R Core Team 2013). To analyze CRC behavioral responses to opposite sexes and whether these responses depend on sex identity, we performed a binomial test on first choice data. As for residency time (sec) in the olfactometer arms, we modeled those data with a linear mixed effects model, with treatment as a main effect and trial day as a random effect, using the package *nlme*. These analyses allow us to statistically confirm whether we reject or accept the null hypothesis that CRC shows no preference for either arm of the olfactometer. To test for sex-based differences in cuticular hydrocarbon profiles, we performed a PERMANOVA with sex as the explanatory variable. Finally, to visualize the cuticular hydrocarbon data, we used non-metric multidimensional scaling (NMDS) as a way to condense the multivariate data into a 2d representation.

RESULTS

Olfactometer assay.

To examine which chemical cues are important for affecting the behavior of CRC and whether there are sex-based differences in response, we performed behavioral assays using an olfactometer. We measured two variables to gauge behavior, first choice and time spent in the arm. The first choice for female CRC was overwhelming to the opposite sex in the two-choice olfactometer assay. Females chose the male odor source over control approx. 80% of the time ($p<0.001$, $n=24$), while males had no observed preference ($p>0.05$, $n=19$) (Figure 5). Additionally, males on average had double the amount of choices following the initial choice.

Sitona hispidulus females responded positively to scents associated with the males as their residence time in the treatment arm was higher compared to the control arm (Figure 6A). Females spent significantly more time in the olfactometer arm assigned to the male odor source than in the control arm ($p=0.002$, $n=24$). In contrast, male weevils did not exhibit a clear preference for either source ($p>0.05$, $n=19$ weevils) (Figure 6B).

Cuticular hydrocarbon profile.

We used GC-MS to characterize contact chemicals that can contribute to conspecific recognition and attraction. Nineteen CHCs were detected (Table 1), including Pentanl-dimethyl, ethanone, and hexane, 2 nitro, which were shared by both sexes. Octadecane, 2-methyl and 24,25-dihydrolanosterol are completely unique to the male sex. Nonadecane, 1,4-Pentadien-3-ol, Eicosane, and 2methyl- are some compounds unique to the female sex. The most dominant compound was Hexane, 2 nitro. As such, we did not detect any significant difference between the sexes in their chemical composition ($p=0.6$, $F=0.516$). In the NMDS plot, the points were not clustered based on sex and were rather spread out with no pattern (Figure 7).

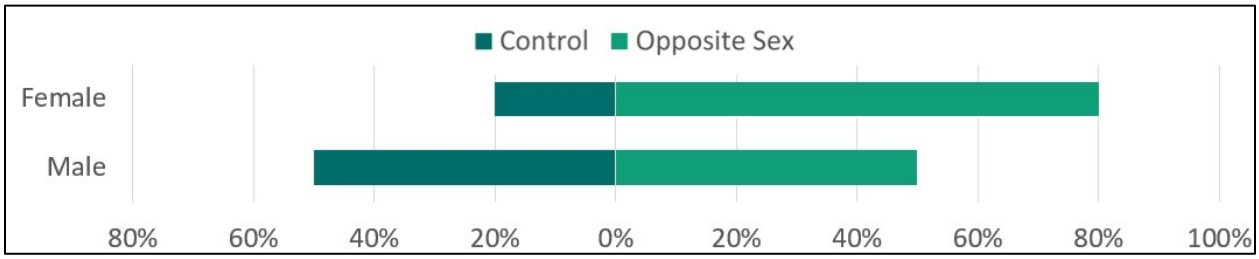

Figure 5. The first choice exhibited by male and female clover root curculio individuals when presented with odor from the opposite sex or a control air stream, measured with a two-choice olfactometry assay. Females chose males over control approximately 80% of the time ($p<0.001$, n=24 weevils), while males had no observed preference ($p>0.05$, n=19 weevils).

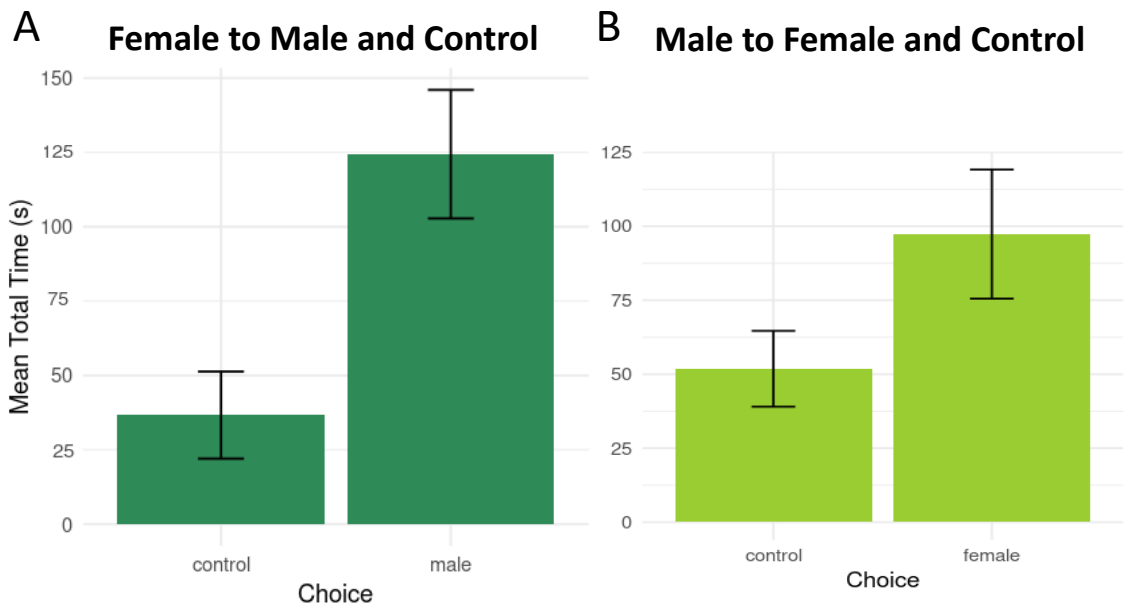

Figure 6. (A) Female clover root curculio spent significantly more time (in seconds) in the olfactometer arm with male odor than control odor ($p=0.002$, n=24 weevils). (B) Males did not spend significantly more time in the female arm ($p>0.05$, n=19 weevils).

Table 1. Cuticular hydrocarbons were detected in bulked sex samples of clover root curculio. Each are listed below, along with their respective peak areas, detected via a Gas Chromatograph Mass Spectrometer. The greater the peak area, the greater the relative abundance of that compound within the cuticular hydrocarbon blend. Each bulked sample contained six weevils in a vile.

	Male B1	Male B2	Male B3	Female B1	Female B2	Female B3
Pentanal, 2,2-dimethyl-	1340623.2390	_	1811723.442	1267302.678	1366508.32	1499369.036
Ethanone 1-(3ethyloxiranyl)	842104.909	860027.365	1183842.792	803976.9567	874258.275	963467.166
Hexane, 2 nitro	986574.28	1002078.56	1389928.51	924532.571	1030769.58	1152827.11
1,5 Heptadien-4one, 3,3,6-trimethyl-	4827270.599	_	_	671438.6258	4960134.326	_
Pentanol 4,4-dimethyl	692811.2	_	_	_	_	_
Tetracosane	416306.9	271404.7	377094.1	_	685236.4	_
Heneicosane, 3-methyl-	202823.4	249861.7	_	374191.4	_	_
Nonacosane, 3-methyl	253809	_	230050.1	_	630347.4	_
Methyl hexadecanoate	_	1346249	_	_	_	_
Cyclopropane, 2-bromo-1,1,3-trimethyl-	_	4852138	6635462	4566739	_	5432763
1-Butene, 2,3,3-trimethyl	_	706791.4	1056247	_	761777.2	_
Octadecane, 2-methyl	_	170832.1	_	_	_	_
24,25-Dihydrolanosterol	_	_	265257.4	_	_	_
1,3-dioxane-5,5-dimethanol, 2-hexyl	_	_	_	441888.1	_	_
Eicosane, 2methyl-	_	_	_	265580.5	_	_
Nonadecane	_	_	_	_	489195.2	_
1,4-Pentadien-3-ol	_	_	_	_	_	890326.9
Undecane	_	_	_	_	_	199638.7
Sulfurous acid, dodecyl-2 propyl ester	_	_	_	_	_	127053.2

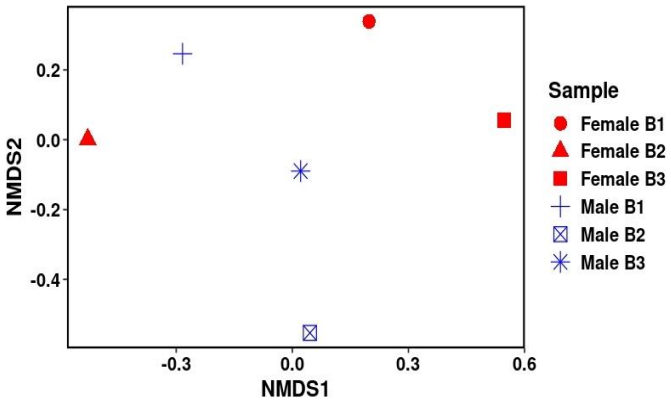

Figure 7. Non-metric dimensional scaling plot of cuticular hydrocarbon profiles of male and female clover root curculio. The closer two points in the plots, the more similar the samples were in chemical composition.

DISCUSSION

In our investigation, we examined the role of semiochemicals in mediating conspecific attraction and mating behaviors in CRC, a soil-borne pest posing significant challenges to producers of alfalfa and other leguminous crops across the United States. By conducting behavioral assays utilizing an olfactometer and complementing them with GC-MS analysis of cuticular hydrocarbons, we sought to elucidate the chemical cues influencing CRC behavior and to explore potential sex-based differences in response. Our findings suggest the presence of a male-produced volatile aggregation pheromone, as evidenced by the strong preference by female CRC for male odors in olfactometer assays. However, our analysis of cuticular hydrocarbon data revealed no significant sex-based differences in the composition of the epicuticular wax layer. These insights into the chemical mediation of mating interactions within the Curculionidae family are integral to the development of sustainable pest management strategies. We propose that leveraging this knowledge could result in the development of semiochemical-baited traps as a cost-effective detection method to alert growers of CRC presence, disrupt mating, and mitigate crop damage.

An overwhelming majority of insects utilize sex pheromones to mediate mate finding. Among these, most have sex-based signaling where the female produces a pheromone. Male-produced chemicals are not distributed evenly among the insect orders, and most are found within the Coleoptera (Mayer & McLaughlin, 1990). Females are expected to perform whichever role in pair formation is less costly (in this case pheromonal signaling versus searching). Searching requires an expenditure of considerable energy and greater exposure to predation and harsher environmental conditions, while pheromonal signaling involves relatively little risk or energy expenditure. Female-to-male attraction only occurs under circumstances that counteract these negative selection pressures by gaining something of value (Thornhill & Alcock, 1983). Species using male-generated pheromones are usually associated with host plants or plant material used by females for feeding or oviposition (Landolt, 1997). Our data for the more unusual case of male-

produced pheromones in *Sitona hispidulus* coincides with what was previously assumed for similar Curculiod beetles, as well as other *Sitona* species such as *S. lineatus* and *S. discoideus*.

The vast majority of the pheromones released by males are aggregation pheromones. It is referred to as an aggregation pheromone because it results in both sexes arriving at the mating site, usually at a food or oviposition site. The male-produced aggregation pheromone for *S. lineatus* was found to attract both males and females (Blight & Wadhams, 1987). However, it is still unclear whether the male-produced pheromone for *S. discoideus* is an aggregation or a sex pheromone, but only females responded in behavioral assays and sexual dimorphism exists in olfactory neurons (Unelius et al., 2013). Our results for CRC share similar uncertainties as only females responded. To confirm male production and aggregation it would be necessary for us to continue olfactometry behavioral assays to observe male-to-male attraction.

It is also imperative that we conduct mating behavior experiments at the right time. No other previous experiments have been performed on CRC, and as such, we have to guess the best time for experiments by observing adult population activity in the field. Previous research has observed lower activity in the spring in the Intermountain West than in the generalized life cycle, and as such we can infer that there is less mating and that many males are not producing aggregation pheromones at that time. Studies should be done to compare pheromone and mating activity to better understand when best to set out traps, as previous work in a related species (*S. lineatus*) has revealed considerable variation in weevil captures across seasons for chemical-bated lures (Bandeira et al., 2021).

To develop effective signaling, it may also be important to investigate host plant and rhizobia volatiles. The soil zone surrounding the roots of plants (rhizosphere) provides a very attractive environment for a vast number of organisms since plants can release up to 20% of the photosynthetically fixed carbon by roots (Barber & Martin, 1976). Multiple volatile interactions occur in the soil between plants, microbes, and insects, among other groups (Wenke et al., 2010). These plant volatiles in relation to male pheromones need to be considered, as the attractiveness of the male-emitted aggregation pheromone in beetles is usually enhanced by host plant volatiles mixture (Giblin-Davis et al., 1996; Pureswaran & Borden, 2005; Wibe et al., 2014). This may be extremely important to consider for *Sitona* weevils, as olfactometry neurons that sense plant and pheromone compounds can be collocated in the same sensillum (Park et al., 2013). At the nervous system level, this could indicate an interactive role in response to different ligands. For *S. lineatus*, plant volitales emitted by host plants were not attractive on their own (St Onge et al., 2018). Other recent studies have shown highly specialized antennal olfactory receptors for pheromone and specific host plant volatiles in *Sitona* species. *Sitona discoideus* can distinguish between the stereoisomers with high sensitivity, and selectivity suggesting a multicomponent communication system (Park et al., 2020). For future work that can directly identify antennal responses to semiochemicals, I have developed an apparatus for measuring electroantennograms in *Sitona hispidulus* (see Appendix A).

Once proper chemical identities and ratios are developed, the male sex attractants will be useful in control programs for CRC through mass trapping or female annihilation strategies. Insect

capture in pheromone-baited traps can be influenced by several factors, including trap design (e.g., type, color, height. In most agro-ecosystems the organisms that feed on plant roots have an important impact on crop yield and can impose tremendous costs to farmers. Similar to aboveground pests, they rely on a broad range of chemical cues to locate their host plant. In their turn, plants have co-evolved a large arsenal of direct and indirect defense to face these attacks. For instance, insect herbivory induces the synthesis and release of specific volatile compounds in plants. These volatiles have been shown to be highly attractive to natural enemies of herbivores, such as parasitoids, predators, or entomopathogenic nematodes. So far few of the key compounds mediating these so-called tritrophic interactions have been identified and only few genes and biochemical pathways responsible for the production of the emitted volatiles have been elucidated and described. Roots also exude chemicals that directly impact belowground herbivores by altering their behavior or development. Many of these compounds remain unknown, but the identification of, for instance, a key compound that triggers nematode egg hatching to some plant parasitic nematodes has great potential for application in crop protection. These advances in understanding the chemical emissions and their role in ecological signaling open novel ways to manipulate plant exudates in order to enhance their natural defense properties. The potential of this approach is discussed, and we identify several gaps in our knowledge and steps that need to be taken to arrive at ecologically sound strategies for belowground pest management (Hiltpold & Turlings, 2012), and placement), lure type, pheromone dose, and environmental conditions during the trapping period (Reddy et al., 2018). The next steps for practical application would involve fieldwork testing the efficacy of the traps to identify the types that would be most optimal. It is important to consider the type of trap and where the trap is placed. For *S. lineatus*, St. Onge et al. (2018) showed that pitfall traps baited with pheromone caught significantly more *S. lineatus* adults than the solo cup, yellow cone, yellow bucket, or green unitrap designs. Reddy et al. (2018) support the results of St. Onge et al. (2018), indicating that pitfall traps are likely the best trap design for monitoring *S. lineatus* adults. They found that ramp traps were as effective as pitfall traps but they are comparatively more expensive and difficult to use. It would also be pertinent to investigate how many pitfall traps would be necessary for control of a field and where to place them best. For example, *S. lipeatus* traps placed at the southern part of a pea field caught nearly twice as many as traps placed at the northern part (Reddy et al., 2018), and higher populations were found in southern compared to northern parts of the field (Quinn et al., 1999). These results could be expected with the knowledge that adults prefer sites in a field with higher temperatures and sunlight. Additional mark and recapture studies may also be needed to determine the maximum radius of attraction to optimize the number of traps and lures required for a given cropping area. It will also be worthwhile to determine the pheromone threshold levels for *S. lineatus*.

Taken together, optimization of a trapping system for *S. hispidulus* would at first be a useful tool in providing information for monitoring and for precise assessments of adult emergence and timing of the control measures, which could then lead to the development of traps that can be more widely used for mitigating crop damage. Work in this thesis has taken the first steps to inform future work for IPM strategies and for improving alfalfa harvests. Our long-term goal is to improve

the ability of producers to manage belowground pests, in turn enhancing yields and the profitability of forage crops.

CONCLUSION

Clover root curculio males likely produce a volatile aggregation pheromone important for mating behavior. Exploration of potential attractive chemicals revealed that females are highly attracted to the male odor, but what component(s) within remains to be investigatedCoupled GC-MS analyses of cuticular hydrocarbons suggest a limited role as contributors to mate recognition and attraction, as there was no sex difference in their epicuticular chemical blends. Future experiments will explore potential key chemicals by measuring the activation of the antenna directly using an electroantennogram. Collectively, these data will contribute to the development of a sustainable monitoring and trapping system for the management of this species.

REFLECTION

Embarking on my Honors Capstone Project investigating the biology of clover root curculio has been a journey that has significantly enriched my undergraduate experience. I feel that this project has allowed me to experience this field and how the start-to-finish process of a project is conducted. Even now, this project is not over, and I am expected to present at the Ecological Society of America Conference this Summer 2024. There are so many ideas and steps that I would want to do to continue, as well as hopefully publish a paper in the process. This project not only fulfilled my Honors academic requirements, but also provided a platform for research experience with the broader goal of serving the agricultural communities of Cache Valley and the Intermountain West.

Throughout this endeavor, I have encountered numerous challenges and triumphs, each contributing to my growth as a researcher and individual. One of the most major setbacks happened a year before the official project began. This was designed to be an experiment I would work on in the Fall of 2022 and 2023. However in the Fall of 2022, I got into a major car accident resulting in a neck injury and a concussion which prevented me from performing field work or any microscope or online work. As stated in the paper, the window for behavioral work in this system is limited. As such, I had to be adapt, be resilient, and adjust plans and expectations for the second year of research.

Data collection and fieldwork have always been what I have been previously part of. One of the most valuable aspects of this project has been the opportunity to engage in the experimental design part of the hypothesis-driven research. By collaborating closely with my mentor, I have delved deep into discussions on hypothesis development and experimental design, honing my skills in making informed decisions for effective research strategies. Additionally, my thorough review of relevant literature has further enriched my understanding of curculio biology and guided the direction of my experiments. Every decision and variable of our experiment has a reason and must be backed by literature and reason, which additionally, we must be able to convey to others.

I have also had to learn the relationship between what you want to do and what is feasible. There are finances and manpower to consider when designing a project. The support from an Undergraduate Research and Creative Opportunities (URCO) scholarship was instrumental in providing me with access to equipment and facilitating my learning of new experimental techniques.

I loved that not only was I able to do the field and laboratory work associated with this project, but I had to train my small team of people. I was able to learn management skills which have been integral to my learning experience. Building upon my prior research experiences in the Schaeffer Lab, I honed my laboratory skills, particularly in working with insects and specialized equipment such as electroantennograms and Y-tube olfactometers. I was happy to have people helping me, however, we unfortunately didn't get the extra help until a week or two before the weevils started dying. We also only had so much equipment to accommodate one assay running at a time, meaning that we had to manage who was doing what, which often resulted in me working late from 3 -10 pm. Additionally, the electroantennogram is a finicky and very precise instrument

that no one in our lab had used, and there were not many tutorials available for a beetle as small as mine. Most experience has been with bees, which required an entirely different electrode and setup. As such, we spent considerable time and effort trying to develop an effective protocol for working with this species; a feat achieved toward the end of their mating season this past fall.

Effective data management and analysis are paramount in scientific research, and this project has provided me with a decent amount of data to organize. I have learned the importance of recording metadata and employing software tools like R for data management and analysis. I initially started with my notebook, which is needed but can't be the only place where the is data stored. It was interesting to learn how data needs to be presented to be used in R and how best to represent the data while sharing it.

Enhancing my scientific communication skills has been a significant focus of this project. From crafting project proposals and reports to presenting findings at conferences, I have honed my ability to communicate complex scientific concepts clearly and concisely. This spring, I presented at the Annual Utah State Student Spring Research Symposium. I also plan to attend the Annual National Meeting of the Ecological of America, and my abstract has been accepted as of the 25th of April. This project has provided me with invaluable opportunities to share my research and engage with fellow scientists.

Looking ahead, the impacts of this capstone project extend far beyond my undergraduate education. As I aspire to pursue graduate studies in Ecology, the skills and knowledge gained from this project will form the foundation for my future endeavors. The opportunity to immerse myself in the intricate relationships between plants, microbes, and insects through the study of chemical signaling is both intellectually stimulating and personally fulfilling. Furthermore, attending conferences and networking with potential graduate advisors will be pivotal in shaping my academic and professional trajectory.

In conclusion, my Honors Capstone Project has been a culmination of academic rigor, personal growth, and a commitment to serving agricultural communities. By achieving the learning outcomes outlined by the Honors program, this project has not only added substantial value to my undergraduate education but has also laid the groundwork for future research endeavors and a fulfilling career as a scientist.

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AUTHOR BIOGRAPHY

Kristin Leutgeb, an Honors student, is graduating from Utah State University (USU) with a Bachelor's degree in Biology (emphasis in Ecology and Evolution), along with a Chemistry minor. During her tenure at USU she earned the Undergraduate Research and Creative Opportunities Grant from the Office of Research to pursue research in the Schaeffer Lab. While working in the lab she developed and conducted her own research project, as well as aided other collaborative projects. In the process, she gained valuable skills in field and greenhouse work, insect beheavior, greenhouse work, and microbial and molecular biology. Also while at USU, Kristin went on a study abroad trip to Costa Rica to do research on soil respiration and its contribution to global warming in the tropics. Afterwards she worked as a biogeochemical lab technition for Dr. Jessica Murray. In her free time she spends equally as much time in Logan canyon as she does for work and for pleaser rockclimbing, mountainbiking, skiing, snowboarding, and hiking. When she is inside her main goal is to finally, in all their years of dating, beat her fiancé at MarioKart. Kristin plans on to attend graduate school to continue studying plant-microbe-insect interactions. Putting her hard work and determination into a project combining all her learned skills over the years in soils, plants, insects, and their combined respones to global warming.

APPENDIX A. FUTURE METHODS ELECTROANTENNOGTRAM

A novel procedure was developed for CRC, and preliminary data was collected to test CRC response to various plant volatiles and related-species stimuli. Antennal response decreases over time, and as such, a control chemical must be used at various time intervals after sample collection to properly scale the signal intensity. One of our aims is therefore to find a chemical detected by both males and females to use as an equalizer for response intensity analyses. Mount techniques were adapted from other clubbed antennae and were tested on 5 female weevils and 2 male weevils (n=7). Selective responses were obtained from 3 females, while indiscriminate responses were obtained from 2 females and 2 males, indicating an improper connection.

Electroantennogram

The electrophysiological assay determines which volatile chemicals illicit a neural response and should therefore be further tested with behavioral methods. In the assay, responses of male and female CRC adults are measured using an electroantennogram (EAG) (Ockenfels Syntech GmbH, Germany; internal gain 10×). To identify semiochemical compounds that elicit responses, we first dissected the weevil under a low-power stereo-microscope and excised the head at the separation from the next segment using micro-scissors. The head was then mounted on the micropipette tip because the very short club-shaped antenna characteristic of beetles cannot be excised from the head without causing damage to the antenna. (Figure 8). Micropipettes were pulled in lab using a bench top glass blower (Articulating Air Gas Torch). The tip was broken off the micropipettes using micro forceps to obtain tips that were about the diameter of the antenna. The micropipettes were then filled with with electrode gel (Parker Laboratories, Fairfield, NJ, USA) and connected to the electrode, being careful not to create any air bubbles that disrupt the electrical circuit. The club of the antenna was placed against the end of the micropipette without cutting off any segments from the tip (which is often done with other types of antennae). The indifferent electrode was placed inside the head. Micropipettes were replaced with each new antenna tested.

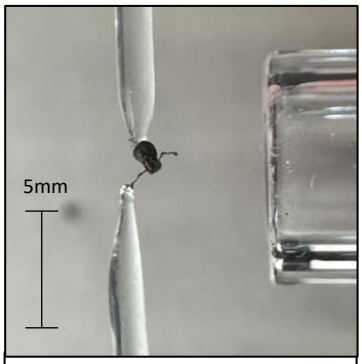

Figure 8. Electrodes connected through a micropipette filled with electrode gel. Head mount performed to minimize damage associated with other known procedures.

The prepared probe was mounted in humidified constant air and near a volatile sample tube for 2-3 min until the signal stabilized. Sample puffs were delivered at 1-min intervals to allow antennae to re-equilibrate after exposure. Antennal responses, which indicate detection, were recorded with Autospike software (Syntech, v.3.9). Maximum responses within a 2 sec window after sample puff were selected and recorded as absolute values. Odor samples were prepared using 6 mm grade AA discs (Whatman, Maidstone, United Kingdom), loaded with a 0.4 μmol solution containing an individual metabolite. The carrier solvent (pentane or hexane, depending on

metabolite solubility) was allowed to evaporate for 2 min, with the filter paper then placed within a Pasteur pipette, trimmed to 6.5 cm and sealed with Parafilm (Bemis NA, Neenah, WI, USA).

With this setup (Figures 8 and 9), odors were directed through the electroantennographic apparatus containing the mounted antennae and probe using both a 0.5 sec pulse flow (300 ml min−1) and a humidified continuous flow (125 ml min−1). To account for variability between individuals, as well as over time since antennae dehydrate, samples will be bracketed by blanks (20 µL carrier solvent corresponding to sample and bioassay disc) and standard stimuli (to be determined) dissolved in corresponding carrier solvent and bioassay disc). For each sample puff, mean blank responses will be subtracted from sample and standard stimuli responses, with the sample antennal response values normalized to the mean blank-corrected standard stimuli response.

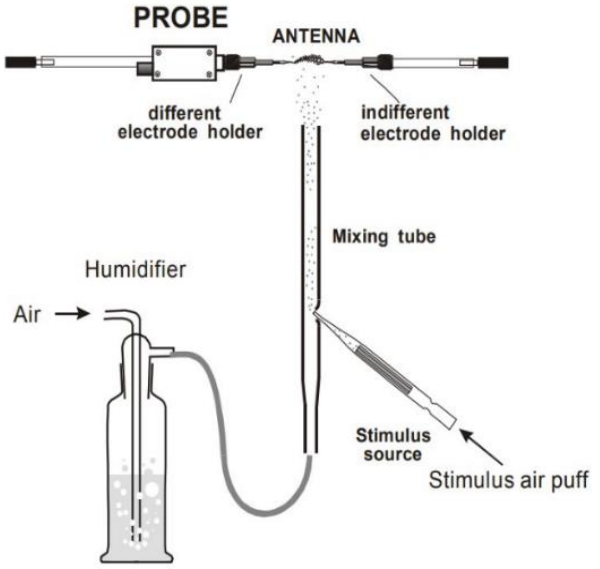

Figure 9. Electroantennogram apparatus to stimulate the antenna with various alfalfa, rhizobia, and CRC volatiles.