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Research

Sensory and behavioral responses of braconid parasitoids to changes in volatile emissions induced by wheat stem sawfly (Hymenoptera: Cephidae) larval feeding in winter wheat and smooth brome

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The wheat stem sawfly, *Cephus cinctus* Norton, is a major pest of cultivated wheat (*Triticum aestivum* L.) and other cereals in North America. The native congeneric parasitoids *Bracon cephi* (Gahan) and *B. lissogaster* Muesebeck are important biocontrol agents and play a crucial role in managing wheat stem sawfly outbreaks and damage. Smooth brome grass (*Bromus inermis* Leyss) has been found to be an effective wheat stem sawfly sink and parasitoid source when grown in areas neighboring wheat fields in Montana. To better understand the ecology of the system, we investigated both the wheat stem sawfly-induced volatile organic compounds produced by smooth brome and winter wheat, and the electrophysiological and behavioral response of *B. cephi* and *B. lissogaster* to the collected volatiles via coupled electroantennography and gas chromatography-flame ionization detection. Volatile concentration analysis indicated significantly increased production of (*Z*)-3-hexenyl acetate, 6-methyl-5-hepten-2-one, and (*E*)-2-hexenal in wheat stem sawfly-infested smooth brome, and elevated production of 6-methyl-5-hepten-2-one in infested smooth brome and winter wheat when compared to their uninfested counterparts. Both *B. cephi* and *B. lissogaster* exhibited significant electrophysiological and behavioral response to (*Z*)-3-hexenyl acetate, 6-methyl-5-hepten-2-one, and hexahydrofarnesyl acetone. Our results provide important evidence supporting habitat management recommendations that will enhance the effectiveness of biological control, contributing to more sustainable agricultural practices and the preservation of vital ecological functions.

Keywords: biological control, parasitoid ecology, behavior, chemical ecology.

Introduction

The wheat stem sawfly, *Cephus cinctus* Norton (Hymenoptera: Cephidae), is a grass-mining sawfly native to North America (Criddle 1922, Ainslie 1929, Wallace and McNeal 1966, Lesieur et al. 2016, Weaver 2023). Adult female wheat stem sawfly use their saw-like ovipositor to deposit their eggs within the lumen of stems, where the hatched larvae feed on parenchymal tissue (Roemhild 1954, Holmes and Farstad 1956). Economic damage manifests in the form of kernel weight reduction due to larval feeding (Delaney et al. 2010, Beres et al. 2011a, 2011b), and stem toppling caused by larval girdling during overwinter chamber preparation that results in unrecovered wheat heads (Holmes and Peterson 1960, Nansen et al. 2005, McCullough et al. 2020).

Bracon cephi (Gahan) and *Bracon lissogaster* Muesebeck (Hymenoptera: Braconidae) have been consistently observed targeting wheat stem sawfly larvae feeding within wheat stems (Nelson and Farstad 1953, Morrill et al. 1998, Runyon et al. 2001). These 2 highly host-specific ectoparasitoids, native to North America, have demonstrated the ability to provide biological control by causing significant and irreplaceable mortality in wheat stem sawfly populations (Peterson et al. 2011, Buteler et al. 2015) and can significantly reduce wheat stem sawfly damage and impact on crop

yields (Buteler et al. 2008, Bekkerman and Weaver 2018, Adhikari et al. 2019, Rand et al. 2020). Wheat stem sawfly is the only known host (Runyon et al. 2001), although hyperparasitism can occur between congeners as well as intraspecifically (Nelson and Farstad 1953, Davis et al. 1955).

Smooth brome (*Bromus inermis* Leyss) is a Eurasian cool-season, non-native, rhizomatous grass, and could potentially be useful as a trap crop to manage wheat stem sawfly (Criddle 1922, Seamans 1928, Farstad and Jacobson 1945, Beres et al. 2011). The grass is found along roadways and within coulees that border much of Montana's "golden triangle," an area of central and northern Montana with intense cereal grain production. The grass is found in all Canadian provinces and all but 3 US states (USDA-NRCS 2002), including all major wheat-producing states. Due to its relatively low agricultural value and propensity to form monocultures that outcompete many native grasses, smooth brome is considered variably as problematic in some habitats (Carlson and Newell 1985, Willson and Stubbendieck 2000, Dillemuth et al. 2008, Grant et al. 2020). Yet, it still holds considerable ecological and economic value due to its prolonged growing season and late senescence, as well as wheat stem sawfly larval antibiosis properties (Otfinowski et al. 2006, Rand et al. 2024, Strand et al. 2024).

Parasitoids of herbivorous insects operate within a complex ecological framework. Consequently, their physiology and behavior are shaped by interactions across multiple trophic levels, including their herbivorous hosts and the plants those hosts consume (Price et al. 1980). Natural enemies rely on information from these trophic levels to make foraging decisions, with chemical cues playing a crucial role. The foraging behavior of parasitoids typically involves several distinct stages: host habitat location, host location, host recognition, and host acceptance (Vinson 1998), with host and habitat location often involving the use of nonvolatile and volatile chemicals from the host or host plant (Vet and Dicke 1992, Turlings and Benrey 1998, Steidle 2000). Numerous studies have demonstrated that herbivore-injured plants produce specific blends of odors that can be attractive to certain insect predators and parasitoids (Vet and Dicke 1992, Bernasconi et al. 1998). In agricultural settings, internally feeding grain beetles and their associated parasitoids became a model system used to understand how parasitoids use chemical cues to locate hosts that feed concealed inside host plant structures (Steidle et al. 2001), as well as the previously mentioned studies from foliar feeding species.

Volatile organic compounds (VOCs) in plants are a diverse group of organic chemicals that plants produce and release into the atmosphere to communicate with other organisms in their environment. These compounds can volatilize easily at room temperature due to their high vapor pressure. Volatile compounds are usually lipophilic liquids with high vapor pressures, allowing these small molecules to easily cross membranes and be released into the atmosphere or soil if no diffusion barrier is present (Dudareva et al. 2006). Significant research has been conducted on the production of both passive (Hamilton-Kemp and Andersen 1986, Gianoli and Niemeyer 1997, Piesik et al. 2008) and induced volatile chemicals (Perez 2009, Delaney et al. 2013, Castelyn et al. 2015) by wheat. Subsequent studies evaluated the electrophysiological and behavioral responses of insects like wheat stem sawfly (Cossé et al. 2001, Piesik et al. 2008) and its 2 associated specialist braconid parasitoids (Perez 2009, Bhandari 2020) to volatiles from uninfested and infested wheat and other host plants. Bhandari (2020) found that *B. cephi* and *B. lissogaster* preferred wheat stem sawfly-infested smooth brome over wheat stem sawfly-infested spring wheat, leading us to question whether the same was true for smooth brome versus winter wheat. Because it is planted in the fall and experiences winter vernalization, winter wheat matures and begins stem elongation earlier in the summer, making it susceptible to wheat stem sawfly infestation sooner.

A better understanding of the benefits that unmaintained, seminatural environments offer to agroecosystems is crucial for enhancing conservation biocontrol in intensified monocultures. Part of this is understanding the chemical-based mechanisms that govern these processes—at the level of sensory responses and subsequent elicited behaviors. Hence, we aimed to characterize and compare the VOC profiles of both winter wheat and smooth brome, and to analyze the electrophysiological and behavioral responses of wheat stem sawfly parasitoids to these volatile blends. Our findings may help inform future integration of the ecosystem services provided by parasitoids and smooth brome in IPM programs in dryland wheat production settings that are challenged by wheat stem sawfly.

Methods

Plant Culture

Smooth brome ('Manchar') was grown from transplants harvested from a previously established smooth brome patch at the Arthur H. Post Agronomy Farm (43°38′19.39″N, 116°14′28.86″W). Immediately following collection, plants were placed into 7 × 35 cm lightweight dee pot cells (Stuewe & Sons Inc., Tangent, Oregon) and moved into greenhouse space at the Montana State University Plant Growth Center (MSU-PGC). Winter wheat ('Yellowstone') was grown in 2 × 20 cm conical pots. The soil used for both plant species consisted of MSU-PGC soil mix (equal parts sterilized MSU-PGC silt loam soil and washed concrete sand with Canadian sphagnum peat moss incorporated) and Sunshine Mix 1 (Canadian sphagnum peat moss, vermiculite, perlite, and Dolomite lime) in a 1:1 ratio. Smooth brome and winter wheat received fertilization once weekly using Jack's Professional Water-Soluble Fertilizer (20-20-20) (J.R. Peters Inc., Allentown, Pennsylvania). Supplemental light was supplied from model MVR1000/C/U GE Multi-Vapor Lamps (GE Lighting, General Electric Company, Cleveland, Ohio) under a photoperiod of 16:8 (L:D) h at 22 °C and 20% to 40% RH. After wheat plants reached Zadoks 12 (Zadoks et al. 1974), they were moved into vernalization storage (8:16 (L:D) h at 4 °C and 20% to 40% RH) for 6 wk to experience the length of dormancy needed for successful stem and flower development. After the 6-wk period, stems were transplanted into 7 × 35 cm conical pots.

Insects

Wheat stubble collected the year prior to each experiment was used to rear adult wheat stem sawfly. Wheat stubble was collected from Big Sandy (48°10′39.14″N, 110°6′50.62″W) and Three Forks (45°59′33.38″N, 111°37′7.42″W), Montana. Wheat stem sawfly-cut "stubs," or the basal root and stem remaining after wheat stem sawfly cutting, were collected from fields that experienced high levels of wheat stem sawfly infestation and cutting the year prior. The stubs, which contained wheat stem sawfly larvae in diapause, were kept in a refrigerator between 0 and 4 °C for >100 d as required to complete obligatory larval diapause. After diapause completion, stubble was kept at room temperature between 22 and 27 °C for 4 to 5 wk in sealed 70 cm × 35 cm × 20 cm plastic Tupperware bins to allow for larval pupation and adult emergence. Once emergence began, adults were collected daily and sorted in 2-L glass mason jars sealed with filter paper to allow for airflow. Jars containing adults were refrigerated at 6 to 10 °C until they were needed for experiments.

Adults held in refrigeration for more than 48 h were not used for experiments.

Wheat stem residue (non-wheat stem sawfly-cut stem material) containing pupal parasitoids *B. cephi* and *B. lissogaster* were collected at the same time as stubble from wheat fields in Three Forks, Montana (46°1′12.78″N, 111°35′11.70″W) that experienced high levels of wheat stem sawfly infestation the year prior. Stem residue was collected and stored inside 170-L plastic barrel liners and stored in a cold storage room at 4 °C until mid- or late spring of the following year to provide enough time for the insects to complete obligatory diapause. Plastic barrel liners were then removed from cold storage and the residue was placed inside 170-L trash barrels at room temperature and watered to encourage larval development and emergence. Parasitoids were collected daily as they emerged and kept at 6 to 10 °C refrigeration and provided sugar supply until used. Insects kept in refrigeration for longer than 7 d were not used for experiments.

Greenhouse Infestation and Volatile Collection

Grouped winter wheat and smooth brome plants were infested simultaneously to ensure consistent wheat stem sawfly-induced volatiles. Once the first node was detected in the winter wheat (typically around Zadoks 32), winter wheat and smooth brome plants were paired based on maturity. Methods for infestation were adopted from Biyiklioglu et al. (2018). Briefly, plants were infested by placing a transparent plastic cylinder (60 × 3.8 cm) over the main stem of the plant. A 55 to 60 cm bamboo stick was added to the soil within each tube to help prevent toppling of the cylinder. Each cage had 3 holes, as well as an open top, covered with fine 530 µM mesh cloth to allow for air circulation. Four females and 2 male wheat stem sawfly, newly emerged, were introduced into each tube via a small 1 cm opening. After which, the hole was plugged using a cotton ball covered in mesh cloth. Damp "50-50 mix" soil was mounded around the base of each tube to prevent insect escape. Adult wheat stem sawfly were left within infestation tubes for 3 d, during which the plants were not watered or fertilized. Afterwards, the tubes were removed, and any surviving insects, as well as cadavers, were removed. The plants were then returned to normal water and fertilization schedules. For the overall experiment, infestation was undergone 3 d per week for 5 wk. Each replicate included 6 winter wheat plants and 6 smooth brome plants. For each replicate, 4 wheat and 4 smooth brome plants were infested, with 2 of each left uninfested as controls. Infestation tubes were placed around all 12 plants for each replicate.

Volatile Organic Compound Collection

Volatile collection and successive GC-MS analysis were conducted following methods outlined in Weaver et al. (2009). Collections were conducted at Zadoks stage 49—approximately 6 wk after infestation, for a period of 6 h from 0900 to 1500. Collection trials were performed 3 times per week on 12 individual plants at a time. Volatiles were collected using glass volatile collection chambers (VCC) that were 80 cm long and 4 cm in diameter. The top end of each VCC funneled into a threaded port that was 10 cm in diameter. Threaded onto the top of each VCC was an 8-port manifold adapter. Two glass filters (traps) (6.35 mm diameter × 76 mm length; Analytical Research Systems, Gainesville, Florida) with 30 mg of Super-Q absorbent (Alltech Associates, Deerfield, Illinois) were inserted into 2 of the adapter ports. The remaining 6 collection ports were sealed to prevent ambient air exchange and pressure loss. One of the traps was used to collect extraneous volatiles from the initial experiment setup for the first 10 min, whereas the other trap was used to collect volatile compounds emanating from plants beginning 10 min after the system was turned on. A 24/210 threaded-glass joint at the base of each VCC was connected to Teflon tubing that pumped in charcoal-filtered and humidified air pressure regulated by a diaphragm air pump, delivering air at a rate of 1 L/min. A vacuum pump was connected via Teflon tubing to each of the traps to maintain air pressure and flow rate (humidified air at 1.0 L/min) within the collection system. A Teflon guillotine was used at the base of each plant to tightly seal the base of the system and prevent external air from entering. Each plant stem was wrapped with a small piece of cotton prior to the application of the guillotine to further seal the base of the collection system and prevent contamination by ambient and soil-derived volatiles.

After the completion of volatile collection, VOCs were eluted from the glass traps using 200 µl aliquot dichloromethane. Trapped volatiles were eluted slowly by adding dichloromethane and further clearing by using a slow release of nitrogen gas. The eluted samples were collected in a glass insert held within a 1.5-ml crimped top glass vial. Ten µl of nonyl acetate was then added to the eluted samples as internal standard to allow for quantification of compound concentrations. The samples were processed using gas chromatography (GC) on an HP-5MS; 30 m × 0.25 mm, 0.25 µm film thickness column (J and W Scientific, Folsom, California). To analyze, the GC instrument (Agilent 6890; Agilent Technologies, Inc., Santa Clara, California) was coupled to a mass spectrometer (MS, Agilent 5973 instrument). Each sample was injected using the automated system in pulse splitless mode, with the initial pressure set to 82.7 kPa per minute. The inlet temperature of the GC was set to 250 °C while the column temperature was 50 °C for 4 min. The temperature was set to increase at a rate of 5 °C per minute until it reached 160 °C, after which it increased to a rate of 25 °C per minute until it reached 280 °C. The temperature of the transfer line leading to the mass selective detector (MSD) was set at 300 °C. Helium was used as a carrier gas to maintain the flow rate of samples within the column at 1.2 ml/min. The MSD was set in "SCAN" mode running from 50 to 300 m/z. The compounds collected from both the smooth brome and wheat samples were identified by comparing mass spectra and retention times using the National Institute of Standard and Technology library. Peaks and volatiles were analyzed using MSD ChemStation (Agilent Technologies, Inc., Santa Clara, California). Fifteen replicates (sets of 12 plants) were collected in 2022, and 13 replicates were collected in 2023.

Electrophysiology

Electroantennography & Gas Chromatography-Flame Ionization Detection

To test for wheat stem sawfly parasitoid response to compounds in smooth brome and winter wheat, we coupled electroantennographic detection with a flame ionization detector (EAD-GC-FID). Adult female *B. cephi* and *B. lissogaster* parasitoids were prepared by excising the head from the body using fine microdissection scissors. For each specimen, the parasitoid head was mounted on a flat electrode supplemented with a small amount of electrode gel (Spectra 360, Parker Laboratories, Inc., Fairfield, New Jersey) to facilitate electrical conductivity. The electrode was connected to a Type INR-5 micromanipulator (Narishige MN-151, Narishige International USA, Inc., Amityville, New York). The second electrode was also coated with electrode gel and connected to the second manipulator insertion point. Prior to connection, one insect antennae was cut down to remove 3 segments. The cut end was then manipulated

to make contact with the second electrode, completing the circuit. This apparatus and method have been consistently used previously in assessing insect volatile response (Agelopoulos et al. 1999, Perez 2009). Purified, humidified air (20 ml/s) was supplied constantly onto the antennae. The amplifier transferred the information to a computer interface with an EAG version 2.7 software (Syntech NL 2001, Hilversum, the Netherlands). Electrophysiological responses were collected and averaged from 6 *B. cephi* and 8 *B. lissogaster*. Although the sample sizes of 6 and 8 parasitoids are relatively small, a high conservation of chemoreceptors in the stem-mining host of this cryptic system (Gress et al. 2013, Robertson et al. 2018) gives us confidence that the results are representative.

EAD equipment was coupled to a flame ionization detector (FID, Agilent) to test for electroantennal response to volatile stimuli. Volatile samples were run through FID (6890N, Agilent Technologies, Inc., Santa Clara, California) using a 30-m, 250-µm Agilent J&W column (Agilent Technologies, Inc., Santa Clara, California). Each sample was injected using the automated system in pulsed splitless mode, with the internal pressure set to 11.8 kPa. The initial oven temperature of the FID was held at 50 °C for 4 min, after which it was increased by 15 °C/min until the final temperature of 160 °C was reached. The inlet temperature of the FID was set to 250 °C with hydrogen flow set to 35 ml/min. Nitrogen was used as the makeup gas and set to a flow rate of 40 ml/min and a pressure of 400 kPa. Air flow was set to 450 ml/min with a pressure of 550 kPa. Once a run was triggered, both FID output and EAD responses were recorded using EAD Plus (EAD, Santa Clara, California).

Electroantennography Using Synthetic Volatiles

Adult female parasitoid preparation was similar to that described above. Notably different, a single antennae was used for each antennogram, instead of an entire head. Opposite ends of the antennae were connected to the electrodes. For each trial, 10 µl of the test compound was applied to filter paper inserted into a glass pipette connected to controlled airflow (Birkett et al. 2004), which then released an airborne stimulus onto the antennae. A release parameter of 0.4 s was set for the delivery, mixed into the constant flow of air (20 ml/s). The antenna's electrical depolarization was detected, amplified, and recorded by the amplifier and Syntech EAD Pro software (Syntech, Hilversum, The Netherlands) (Bruce and Cork 2001). Five synthetic compounds were used, (*Z*)-3-hexenyl acetate, hexahydrofarnesyl acetone, 6-methyl-5-hepten-2-one (99%), β-ocimene (70%), (*E*)-2-hexenal (99%), and (*E*)-2-hexenyl acetate (98%). All compounds were sourced from Aldrich Chemical Company (Milwaukee, Wisconsin). A single puff of a compound was delivered in every trial, with a new parasitoid used each time. For each insect, hexane was used as a control, establishing a baseline electrical response before and after each test compound. The concentrations of each volatile prior to dilution and number of parasitoids tested for each volatile and dilution series can be found in Supplementary Table 1. We tested parasitoid responses to 3 dilutions of each compound: 0.01, 100, and 10,000 ng. Compounds and dilutions were selected randomly for each trial.

Behavioral Trials

Y-Tube Olfactometer Bioassay

Bioassays were conducted to assess variation in behavioral responses of naive female parasitoids to volatiles from both winter wheat and smooth brome. The volatiles used were a combined mixture of 15 samples collected during our volatile collection experiment. These samples were pooled from plants of the same type and infestation status to ensure that the volatile concentrations were high enough to elicit a response from the parasitoid antennae. This approach effectively mimicked the volatile signature of smooth brome and winter wheat within the Y-tube airstream.

We used a Y-tube system (Analytical Research Systems, Micanopy, Florida) similar to those used by Piesik et al. (2008) and adapted for parasitoids in Cavallini et al. (2023). Y-tubes consisted of a Corning glass tube (28 mm diameter × 300 mm long) that branched at 20-cm along a 120° angle to form the arm of each tube. Each arm extended 3 cm from the junction before becoming parallel, with a total length of 10 cm for each parallel section. Each arm was connected to a diaphragm air pump that delivered charcoal-filtered and humidified air at a rate of 0.2 L/min. Fifty µl of extracted volatile mixture was pipetted onto a small piece of filter paper and placed inside a 46-cm Corning tube with a ground-glass joint at 1 end to connect to the Y-tube, and a threaded-glass joint to receive the air supply. Two of these tubes were used for each trial. Y-tubes were used for a single trial before being exchanged for a fresh, clean tube. After each assay, glassware was cleaned using a non-foaming anionic Liquinox detergent (Alconox Inc., White Plains, New York) in warm water, rinsed with water, and rinsed with the solvents acetone and hexane. Glassware was then placed in a 110 °C oven and baked for a minimum of 2 h before the next use.

For each assay, a single naïve female *B. cephi* or *B. lissogaster* parasitoid was introduced 1 cm into the unbranched end of the Y-tube (Cavallini et al. 2023). To facilitate female parasitoid movement toward the Y-tube junction, a black box containing the Y-tube was set up at a 10° angle with the "Y" end elevated toward a light source. In addition, a 28-cm long metal wire was placed on the unbranched portion of the Y-tube, spanning the length from the introduction point to the branch. Females were placed inside the Y-tube on the top of the wire, 3-cm from the introduction point. Naïve females were randomly assigned to each assay. Females were observed for 2 min and their choice in the olfactometer was recorded. An insect was considered to have made a choice if it showed any directional movement at the end of the Y-tube. If the parasitoids did not make a choice within the 2-min observation period, they were recorded as "nonresponders." Individuals were only used for a single trial. We used a total of 58 *B. cephi* and 68 *B. lissogaster* during these trials.

Due to the inconsistent nature of parasitoid emergence and the short lifespan of their excised heads and antennae, data for both electrophysiological and behavioral experiments were collected over an extended period of several months.

Wind Tunnel Behavior Bioassay

To test female parasitoid behavioral response to independent synthetic volatiles, we used a 150 cm × 50 cm × 50 cm (L × W × H) acrylic box with airflow rates set to 0.2 L/m. Setup was similar to that described in Miller and Roelofs (1978). Briefly, at the end of the wind tunnel, 2 plastic Petri dishes (Fisher Scientific, Pittsburgh, Pennsylvania) were positioned, each featuring a 2 × 2 cm square opening in the center and coated with Tanglefoot on the outer surface. These dishes were mounted 20 cm high and 30 cm apart. Each dish was equipped with a rubber septum, treated and secured by a paper clip, and positioned in the center of the opening. The septum released the compound into the air stream. To attract parasitoids, a 12-V light bulb was placed behind each opening to exploit positive phototaxis. The entire setup was situated in a dark room maintained at a constant temperature of ~27 °C.

Behavioral trials were conducted using pure synthetic compounds that represent those produced by wheat stem sawfly host plants.

The tested compounds were diluted using hexane to obtain the concentrations used for each trial. Compound concentrations can be viewed in Supplementary Table 2.

For each compound tested, female parasitoids were used in groups of 10. In each trial, 10 parasitoids were placed on a platform 25 cm high and 100 cm from the odor source. Ten trials were conducted for each concentration. Due to occasional scarcity of parasitoids, both species, which are naturally sympatric and host-specific, were sometimes combined. For each compound, 10 µl of test solution was applied to 1 septum, with the other treated with 10 µl of hexane as a control. Parasitoids were left in the wind tunnel for 24 h, with counts recorded at 8 and 24 h. Septa and Petri dishes were replaced after each trial, and their positions randomized. Overall, 205 female *B. cephi* and 155 female *B. lissogaster* were used in wind tunnel behavioral trials.

Statistical Analysis

Comparative Analysis of Volatile Collections

The amount of volatile compound present in each sample was calculated in $ng^{-1}\ g^{-1}\ h^{-1}$ after correcting for plant biomass. Untransformed data were used for concentration analysis, while all other analyses used data transformed using a center log-ratio transformation (Compositions, version 2.0-8), a technique commonly used when analyzing multivariate compositional data (Brückner and Heethoff 2017). VOC compositions were compared between samples and treatments using a permutational multivariate analysis of variance (Canberra distance, 999 iterations) test within the vegan package (v 2.6-4) (Oksanen et al. 2011) using R Studio (v4.2.764) (R Core Team 2024). Potential compounds that might contrast volatiles between wheat and smooth brome were further analyzed using ANOVA after fitting a linear mixed model. Each compound was assessed as a response variable with plant type as a fixed effect and replication as a random. Post hoc Tukey's Honest Significant Difference tests were conducted to compare concentration differences between plant types and infestation status.

Seventeen peaks were identified between samples of smooth brome and winter wheat. Compounds for analysis were selected based on MS-identification quality and consistency across volatile collection trials. We used previously defined systemically active volatiles from Piesik et al. (2008) and Perez (2009) to further narrow down our list of volatiles based on both wheat stem sawfly and *Bracon* spp. electrophysiological and behavioral activity.

Synthetic Volatile Electrophysiology

We calculated response ratios using the formula $Ratio = R_{stimulus}/R_{control}$ for each compound to understand how increased volatile concentration impacted insect electrophysiological response. Response ratios for each trial were calculated using the control response (hexane) as a baseline. The data were transformed via a $\log_{10}$ before being analyzed using a *t*-test in RStudio using the Stats package (v4.4.1). To determine the relationship between antennal response and concentration of each compound, we used a simple linear regression analysis.

Behavioral Experiments

A Pearson's chi-square test was used to identify differences in parasitoid response tests to smooth brome and winter wheat volatiles. An alpha level of 0.05 was used for test significance differences in female choices for both *B. cephi* and *B. lissogaster*.

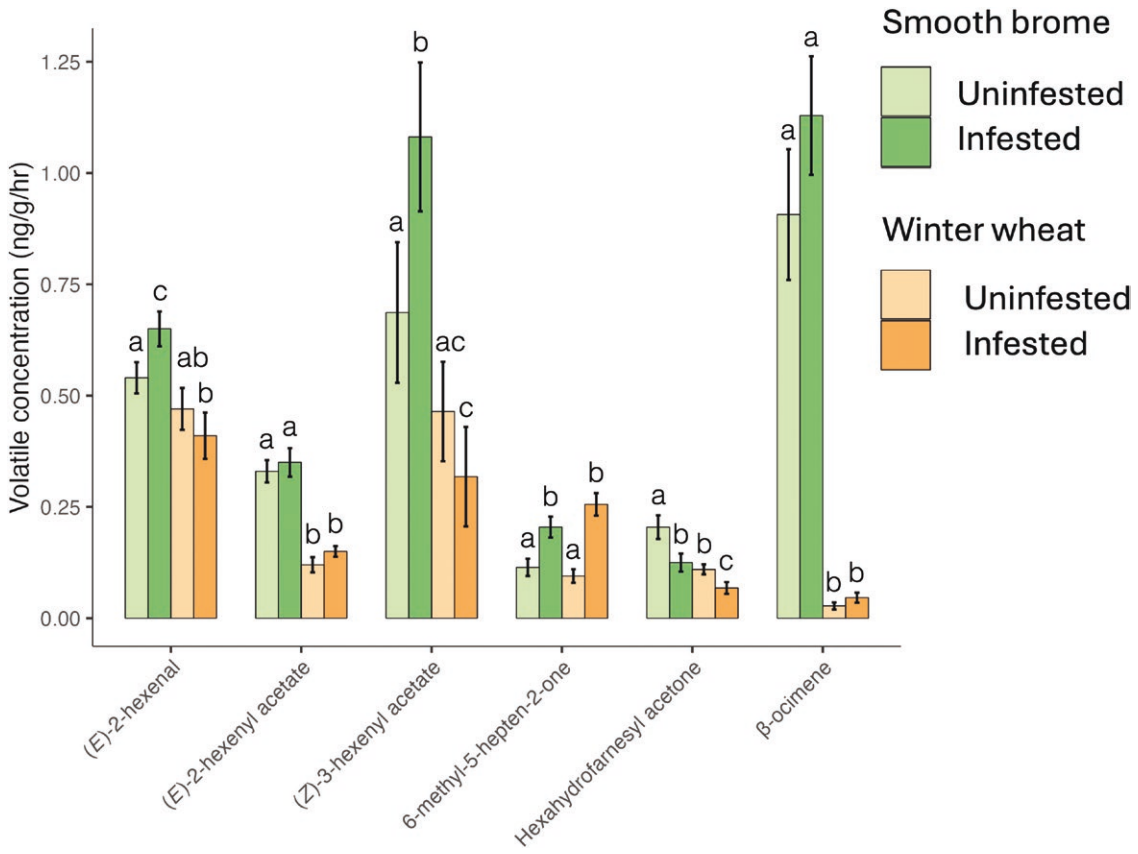

Fig. 1. Comparison of volatile concentrations between wheat stem sawfly-infested and uninfested smooth brome ('Manchar') and winter wheat ('Yellowstone'). Bars within each group with the same letter are not different by pairwise comparison tests, with 5% family-wise significance level.

Results

Collected Volatile Profiles

Overall, smooth brome and winter wheat emitted similar volatiles, but varied in the quantity produced. When comparing smooth brome against winter wheat, irrespective of infestation status, quantities of (*E*)-2-hexenal ($F_{1,122} = 4.08$, $P = 0.03$), (*E*)-2-hexenyl acetate ($F_{1,122} = 18.09$, $P < 0.005$), (*Z*)-3-hexenyl acetate ($F_{1,122} = 11.22$, $P = 0.02$), and β-ocimene ($F1,122 = 41.97$, $P < 0.005$) differed significantly. Concentrations of 6-methyl-5-hepten-2-one ($F_{1,122} = 0.07$, $P = 0.82$) and hexahydrofarnesyl acetone ($F_{1,122} = 0.12$, $P = 0.51$) were not significantly different across plant types.

Quantities of 6-methyl-5-hepten-2-one were significantly greater in both smooth brome ($F_{1,62} = 8.68$, $P = 0.003$) and winter wheat ($F_{1,60} = 13.44$, $P < 0.005$) when comparing infested plants against their uninfested counterparts (Fig. 1). Infested smooth brome yielded significantly greater concentrations of (*E*)-2-hexenal ($F_{1,62} = 7.43$, $P = 0.02$) and (*Z*)-3-hexenyl acetate ($F_{1,62} = 8.45$, $P = 0.001$) when compared to uninfested smooth brome. Infested smooth brome ($F_{1,62} = 14.33$, $P = 0.004$) and winter wheat ($F_{1,60} = 12.93$, $P = 0.002$) both produced lower concentrations of hexahydrofarnesyl acetone when compared to uninfested plants.

Electrophysiology of *Bracon* spp. Parasitoids

Electroantennography-Gas Chromatography-Flame Ionization Detection

To determine which volatile compounds elucidated antennal response from wheat stem sawfly parasitoids, we used EAD-GC-FID to look for electrical depolarization when stimulated by smooth brome and winter wheat volatiles. Significant depolarization from *B. cephi* and *B. lissogaster* were induced for (*E*)-2-hexenal (0.23 ± 0.05 mV, $P < 0.005$), (*E*)-2-hexenyl acetate (0.37 ± 0.06 mV, $P = 0.001$), (*Z*)-3-hexenyl acetate (0.83 ± 0.07 mV, $P < 0.005$), 6-methyl-5-hepten-2-one (0.41 ± 0.05 mV, $P < 0.005$), and hexahydrofarnesyl acetone (0.44 ± 0.05 mV, $P < 0.005$). We consistently saw responses at these locations across all trials, plant types, and parasitoid species (Fig. 2).

Synthetic Volatile Electrophysiology

We used three dilutions (0.01, 100, 10,000 ng) of synthetic volatiles to individually induce depolarization in *B. cephi* and *B. lissogaster*. Consistent and significant dose-dependent depolarization responses of both species of parasitoid antennae were observed to (*Z*)-3-hexenyl acetate, 6-methyl-5-hepten-2-one, (*E*)-2-hexenal, (*E*)-2-hexenyl acetate, and hexahydrofarnesyl acetone. (*Z*)-3-hexenyl acetate and 6-methyl-5-hepten-2-one, (*E*)-2-hexenal, and (*E*)-2-hexenyl acetate produced positive response ratios (ie stronger responses at higher volatile concentrations), while hexahydrofarnesyl acetone produced a negative response ratio (Fig. 3). Full statistical results can be found in Supplementary Tables 2 and 3.

Behavioral Olfactory Experiments

Smooth Brome and Winter Wheat Blends

Adult females responded positively toward air streams containing infested winter wheat volatile blends when given the choice against pure air. *Bracon cephi* chose wheat 67.5% of the time ($\chi^2 = 7.35$, df = 1, $P = 0.006$) and *B. lissogaster* chose wheat 68.5% ($\chi^2 = 7.76$, df = 1, $P = 0.005$) (Fig. 4). Likewise, both species of parasitoids responded positively to air streams containing a volatile blend from infested smooth brome. When given the choice against pure air, *B. lissogaster* chose infested smooth brome volatile blends 80.8% of the time ($\chi^2 = 9.85$, df = 1, $P = 0.002$) and *B. cephi* chose smooth brome 69.4% of the time ($\chi^2 = 3.86$, df = 1, $P = 0.049$) (Fig. 4). Adult female parasitoids from both species preferred air streams containing infested smooth brome volatiles (*B. cephi*: $\chi^2 = 4.23$, df = 1, $P = 0.45$; *B. lissogaster*: $\chi^2 = 5.45$, df = 1, $P = 0.835$) when given the choice against volatiles from infested winter wheat (Fig. 4).

Synthetic Compounds

Behavioral experiments showed that adult females from both species were significantly attracted to (*Z*)-3-hexenyl acetate and 6-methyl-5-hepten-2-one (Fig. 5). Hexahydrofarnesyl acetone generated a more significant behavioral response in both parasitoid species

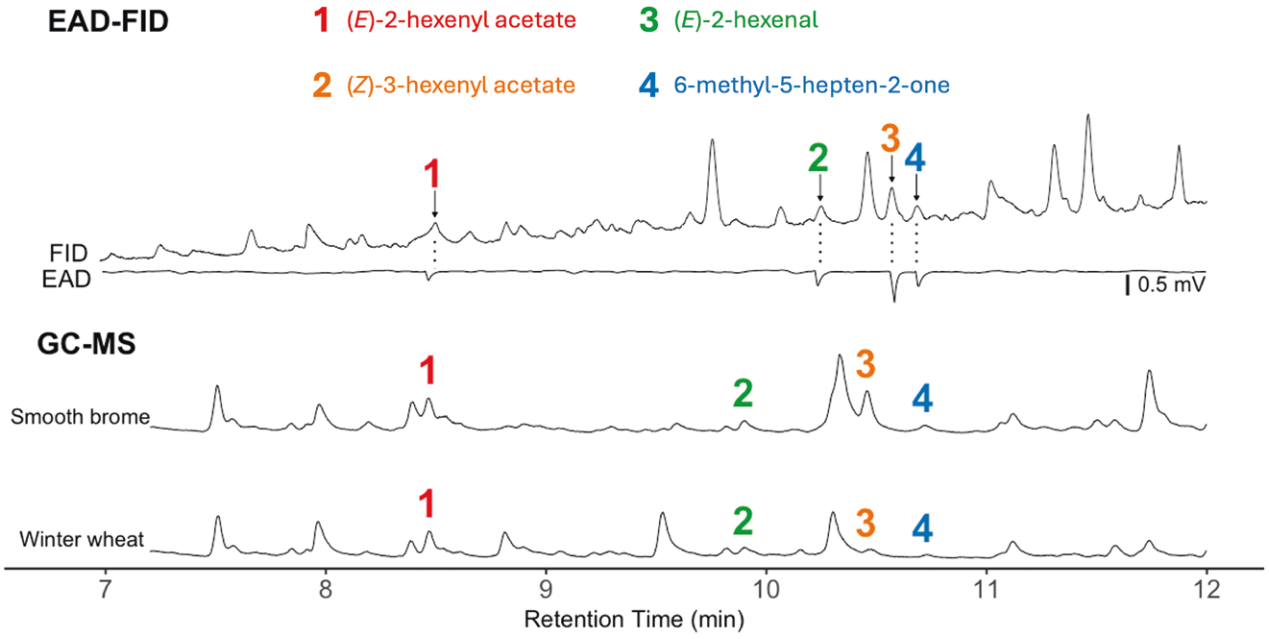

Fig. 2. EAD-GC-FID: electrophysiological depolarization response from adult female *B. cephi* to volatiles blends from smooth brome (*Bromus inermis* Leyss, 'Manchar') sample. Electroantennographic detection and flame ionization detection (EAD-FID) run using volatile blend of 15 collected samples. Lower, GC-MS plot showing infested smooth brome and winter wheat chromatograms to show volatile locations and identifications.

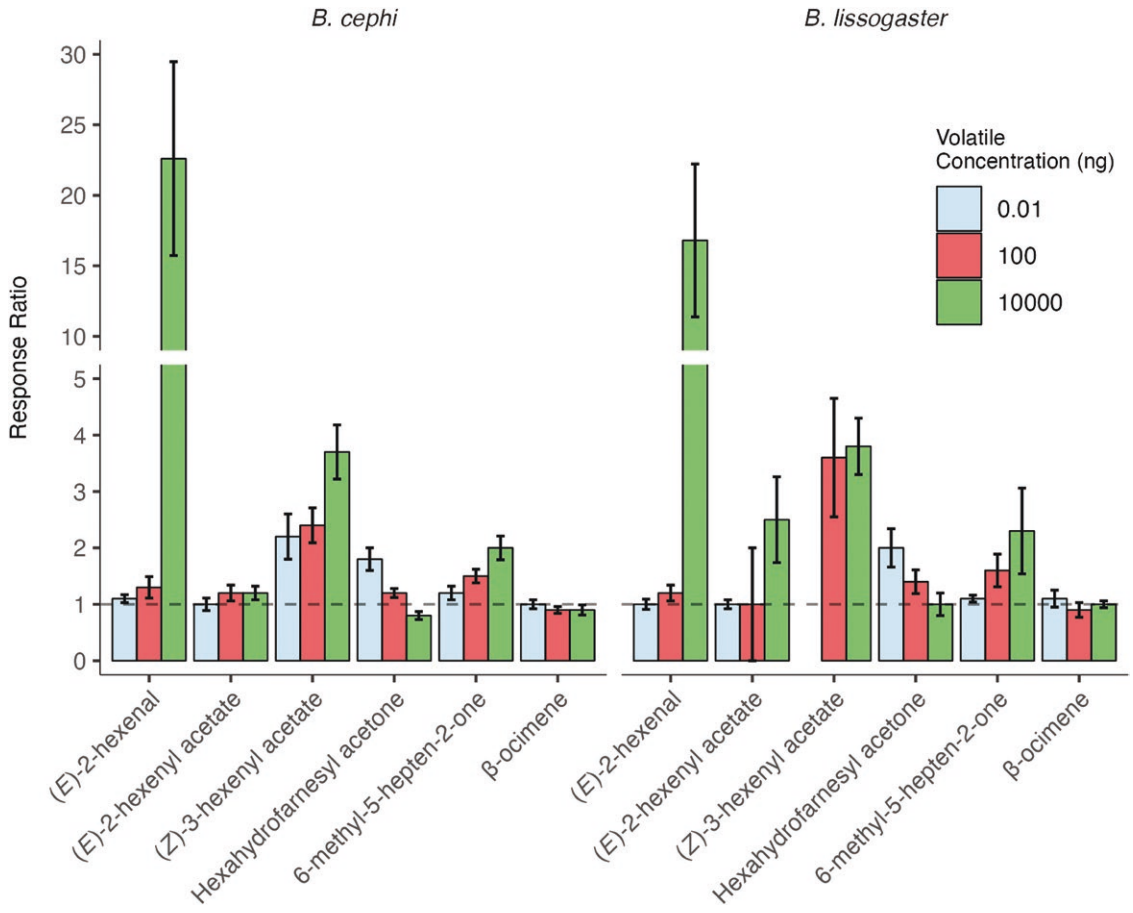

Fig. 3. Electrophysiological response ratios ($Ratio = R_{stimulus}/R_{control}$) of adult female *B. cephi* and *B. lissogaster* to varying concentrations of synthetic volatiles matching those produced by wheat stem sawfly-infested plants. Hexane was used as a control to measure the baseline electrical depolarization prior to each test compound.

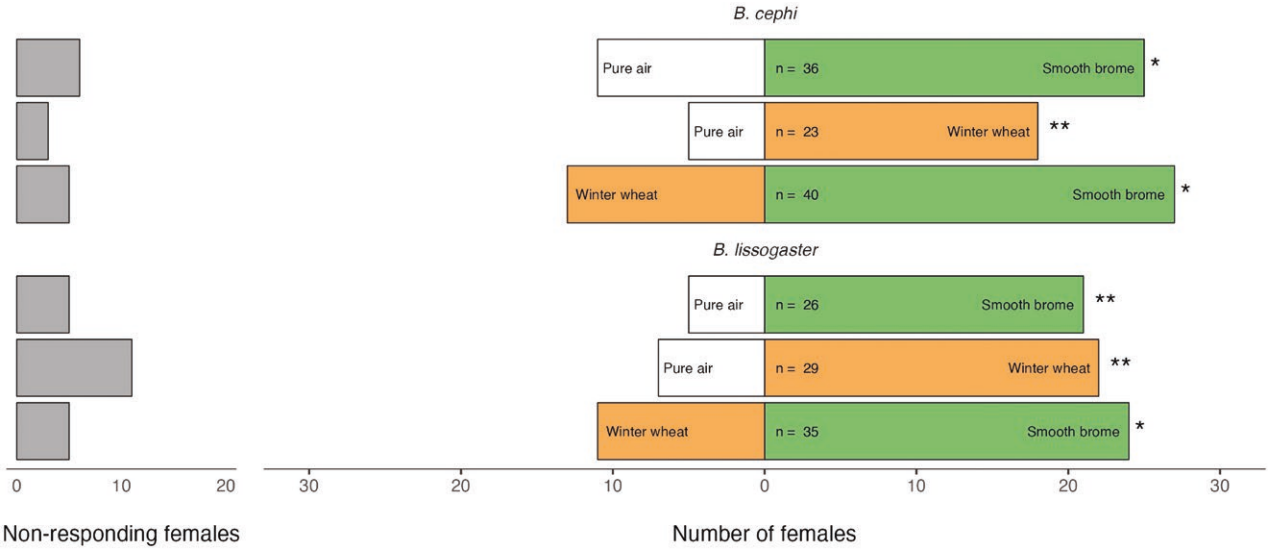

Fig. 4. Behavioral preferences from Y-tube olfactometer bioassay tests for both *B. cephi* and *B. lissogaster* females when choosing between "Pure air", smooth brome ('Manchar') and winter wheat ('Yellowstone') volatile blends. The volatile blends for each plant type were combined volatiles samples from 15 infested plants. Left side bar graphs show the number of nonresponders from each set of related trials. $^{*}P \leq 0.05$, $^{**}P < 0.01$.

at low concentrations (0.3 µg, *B. cephi*: $P = 0.005$, *B. lissogaster*: $P = 0.002$) when compared to high concentrations (9 µg, *B. cephi*: $P = 0.033$, *B. lissogaster*: $P = 0.019$). We found no significant behavioral response at any concentration for (*E*)-2-hexenal or (*E*)-2-hexenyl acetate for either species (Fig. 5; Supplementary Tables 3, 4 and 5).

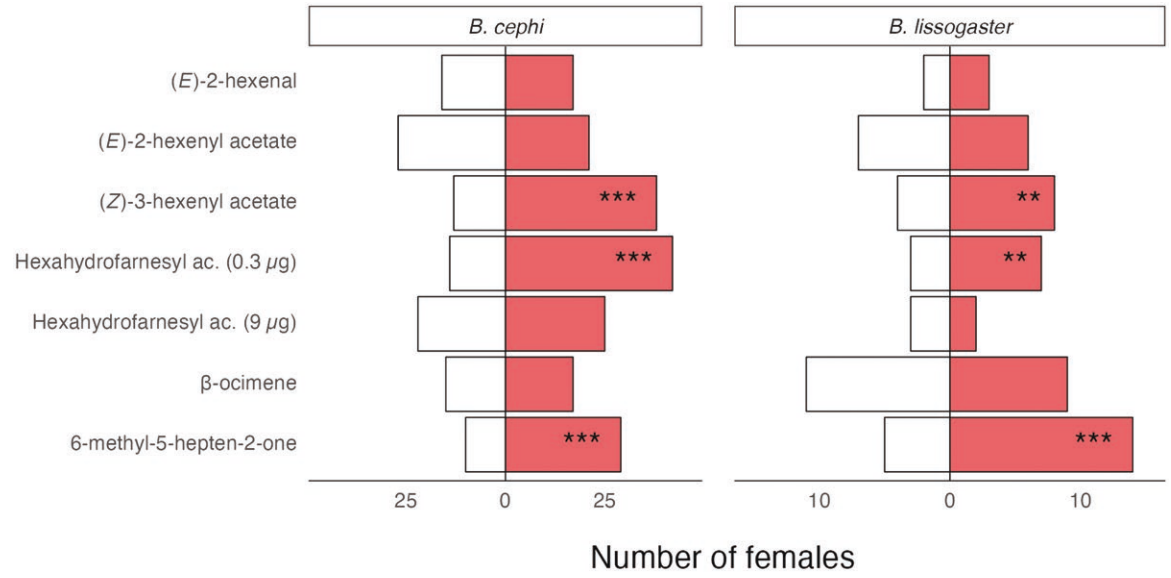

Fig. 5. Behavioral preferences of adult female *Bracon cephi* and *B. lissogaster* to synthetic volatiels matching those produced by infested winter wheat during wind tunnel olfactory trials. Insects chose between "Pure air" (white) and the tested volatile. $^{**}P \leq 0.01$, $^{***}P < 0.005$.

Discussion

Understanding host-seeking behaviors via volatile chemicals is crucial for effectively communicating the benefits of conserving plants that support wheat stem sawfly parasitoids through tri-trophic interactions in seminatural, unmanaged environments in Montana (Strand et al. 2024). Our study builds on previous research showing that adult female wheat stem sawfly parasitoid host plant selection is influenced by olfactory cues (Miller and Roelofs 1978, Buteler et al. 2009). Bhandari (2020) found that adult female wheat stem sawfly parasitoids preferred wheat stem sawfly-infested smooth brome over spring wheat ('Vida') when given the choice. Consistent with these findings, our results showed a clear preference for infested smooth brome over winter wheat ('Yellowstone'), despite the differences in seasonal wheat biology and genetics.

Previous studies have identified 6-methyl-5-hepten-2-one and (*Z*)-3-hexenyl acetate to be potential chemical cues for host finding in the aphid parasitoid *Aphidius ervi* Haliday and in the diamondback moth (*Plutella xylostella* (L.)) parasitoids *Trichogramma chilonis* Ishii and *Cortesia plutellae* (Kurdjumov) (Du et al. 1998, Reddy et al. 2002). (*Z*)-3-hexenyl acetate mediates host and oviposition preference in the fall armyworms (*Spodoptera Frugiperda* (J.E. Smith)) on maize (*Zea mays* L.) (Wang et al. 2023) and is attractive to both generalist and specialized parasitic wasps that target the fall armyworm (Turlings et al. 1993). Our study found that adult female wheat stem sawfly parasitoids exhibit strong electrophysiological and positive behavioral responses to both compounds. Volatiles from both host plants likely include induced responses to wheat stem sawfly-feeding stress that helps to attract wheat stem sawfly parasitoids. Although Piesik et al. (2008) identified 6-methyl-5-hepten-2-one to be a repellent to wheat stem sawfly, our behavioral trials show it to be an attractant for these parasitoids of wheat stem sawfly. Notably, smooth brome did not show increased levels of this compound when compared to winter wheat after a similar duration of infestation, a unique distinction, as we found that smooth brome produces greater quantities of many of the other, electrophysiologically active, volatiles. This distinction may be advantageous in wheat stem sawfly management as gravid wheat stem sawfly females are not deterred from ovipositing while still attracting the parasitoids that use later-instar larvae.

Although β-ocimene was not electrophysiologically or behaviorally active in *B. cephi* or *B. lissogaster*, it was present in greater quantities in smooth brome compared to winter wheat. Previously found attractive to adult female wheat stem sawfly by Piesik (2008), β-ocimene may play a significant role similar to (*Z*)-3-hexenyl acetate and 6-methyl-5-hepten-2-one in explaining why adult female wheat stem sawfly prefer smooth brome over winter wheat.

Both *B. cephi* and *B. lissogaster* responded behaviorally to hexahydrofarnesyl acetone, a compound expressed during wheat ripening (Peck 2004, Miyazawa et al. 2008, Perez 2009). This compound was found in higher concentrations in uninfested plants compared to infested plants. Perez (2009) found lower concentrations of the compound in still green and developing plants and higher concentrations in plants beginning to senesce. Wheat shows decreased photosynthetic activity while suffering wheat stem sawfly tunneling damage (Macedo et al. 2005, 2007, Delaney et al. 2010). This time frame aligns with the behavior of wheat stem sawfly parasitoids, which search for sufficiently sized hosts during a period that extends through plant senescence. During this time, wheat stem sawfly are in late-instar stages, also making them both larger and more damaging to the wheat plant, and a better food source for parasitoid larvae in general (Turlings and Wäckers 2004). Our results showed electrophysiological response and behavioral preference to hexahydrofarnesyl acetone, with significantly stronger responses at lower concentrations. These findings suggest that adult female wheat stem sawfly parasitoids may prefer plants emitting relatively lower concentrations of this compound. This ability to sense minute differences in hexahydrofarnesyl acetone concentrations may help female parasitoids locate high-quality plants infested with wheat stem sawfly larvae, particularly if this preference is part of a specific blend of compounds signaling sawfly stem presence.

Our study provides compelling evidence that volatile chemicals significantly influence the host-seeking behaviors of wheat stem sawfly parasitoids toward two host plants with similar volatile blend profiles. While our experiment focused on these specific species, broader understanding of plant volatile profiles and insect behavioral responses can contribute to more effective pest and biological control strategies. Recognizing how insect behaviors shift in response to plant volatiles can inform conservation strategies aimed

at preserving and managing seminatural habitats that support effective biological control. By identifying behavioral activity, future breeding efforts could focus on developing wheat cultivars that further attract wheat stem sawfly parasitoids, potentially increasing parasitism rates, particularly in fields adjacent to seminatural grasslands. Our results support the argument that the conservation of these unmanaged, seminatural habitats in Montana as refuges can sustain parasitoid populations and enhance biological control (Strand et al. 2024). Previous studies have highlighted the ecological benefits of smooth brome adjacent to wheat fields, providing seasonal refuge for wheat stem sawfly parasitoids (Peirce et al. 2021, Rand et al. 2024, Strand et al. 2024). Our findings further emphasize the importance of strategically conserving and managing these habitats to maximize biological control effectiveness. By identifying the specific volatile compounds that attract wheat stem sawfly parasitoids, we provide essential information for habitat management recommendations aimed at supporting these natural enemies throughout their lifecycles. Future research could explore optimal refuge sizes and configurations to support parasitoid populations, ensuring that landscape-level conservation strategies align with biological control objectives. This integration of knowledge can contribute to more sustainable agricultural practices by maintaining parasitoid populations and promoting long-term ecological stability.

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Author contributions

Jackson Strand (Conceptualization [equal], Data curation [lead], Formal analysis [lead], Investigation [lead], Methodology [equal], Validation [equal], Visualization [lead], Writing—original draft [lead], Writing—review & editing [lead]), Oscar Perez Moya (Conceptualization [equal], Data curation [equal], Formal analysis [equal], Investigation [equal], Methodology [equal], Validation [equal], Visualization [equal], Writing—original draft [equal]), David Weaver (Conceptualization [equal], Funding acquisition [lead], Methodology [equal], Project administration [lead], Resources [lead], Supervision [equal], Validation [equal], Visualization [supporting], Writing—original draft [equal], Writing—review & editing [equal]), Robert Peterson (Conceptualization [equal], Supervision [equal], Validation [equal], Writing—original draft [equal], Writing—review & editing [equal]), and Tracy Sterling (Conceptualization [equal], Supervision [equal], Validation [equal], Writing—original draft [equal], Writing—review & editing [equal])

Supplementary material

Supplementary material is available at *Journal of Insect Science* online.

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