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Racemic Pheromone Blends Disrupt Mate Location in the Invasive Swede Midge, *Contarinia nasturtii*

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Abstract

Swede midge, *Contarinia nasturtii* Kieffer, is an invasive cecidomyiid pest that causes serious losses of *Brassica* oilseed and vegetable crops in the Northeastern U.S. and Canada. Currently, few alternatives to systemic insecticides exist for its management. Because a single feeding larva can render heading *Brassica* crops unmarketable, management strategies that prevent oviposition are needed urgently. Pheromone-mediated mating disruption is a promising management approach for swede midge because it prevents mating and subsequent crop damage. While the swede midge pheromone has been identified, one of the major barriers to using it in mating disruption is the high cost of synthesis. Racemic blends, consisting of natural and non-natural stereoisomers, could be useful for mating disruption because they are cheaper to produce. However, it is not clear whether racemic pheromone blends attract males and/or prevent them from locating and mating with females. Here, we studied the behavior of male swede midge in Y-tube and wind tunnel bioassays to pheromone blends. Specifically, we tested whether males: (1) are attracted to different doses of pheromone, (2) discriminate between blends comprising natural stereospecific or racemic components, or a combination thereof, and (3) are able to locate and copulate with females in pheromone-permeated olfactometers. We found that picogram amounts of pheromone attracted males and prevented them from locating females in y-tube olfactometers. While males were more attracted to stereospecific blends, compared to racemic blends, all blends tested prevented nearly all males mating with females. Therefore, low dose racemic blends may be promising for pheromone-mediated mating disruption.

Keywords Pheromone · Mating disruption · Vegetable pest management · Reproduction · Swede midge · Cecidomyiidae

Introduction

Contarinia nasturtii Kieffer (swede midge; Diptera: Cecidomyiidae) is a small galling fly that is a serious pest of *Brassica* spp. (Brassicaceae) vegetable and oilseed crops in Europe, Eastern Canada and the Northeastern USA (Chen et al. 2011; Hallett and Heal 2001). Larvae feed within the plant meristem, causing deformed and scarred leaves and stems and, in severe cases, can cause complete loss of heads of broccoli, cauliflower, cabbage, and other related *Brassica* crops. Recently, vegetable growers in the US states of New York and Vermont reported up to 100% yield loss of organic kale and broccoli (Y. Chen, C. Hoepting, pers. comm.). No insecticides that are approved for certified-organic production are effective in controlling the midge (Evans and Hallett 2016; Seaman et al. 2014). Due to the severe economic losses inflicted by this pest, some small, diversified organic growers in the region now avoid *Brassica* production entirely (Y. Chen, pers. obs.).

Several aspects of swede midge biology create difficulty managing this pest. The presence of multiple overlapping generations and prolonged crop susceptibility to damage necessitates protection throughout the growing season (Hallett et al. 2009; Stratton et al. 2018). Further, larvae are protected from foliar insecticides within the meristem (Wu et al. 2006). Compounding these challenges is an extremely low damage threshold for vegetables. For example, Stratton et al. (2018) found that a single larva can render a cauliflower plant unmarketable. While some growers use calendar sprays of conventional insecticides to manage

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swede midge, reliance on chemical controls represents a loss of years of progress toward integrated pest management of vegetable pests (Andaloro et al. 1983; Chen et al. 2011). Management approaches that prevent oviposition, such as pheromone-mediated mating disruption, are needed urgently.

Although cecidomyiids can be difficult to manage, certain aspects of their biology and ecology present opportunities for economical pheromone-mediated mating disruption, i.e., the deployment of large doses of synthetic female sex pheromone to interfere with the ability of males to find mates. Adults are very short-lived, with discrete diel periodicity of mating (Bergh et al. 1990; Gagne 1989; Harris et al. 1999; Hodgdon et al. 2018). In previous studies, we found that swede midge males are responsive to pheromones in the morning (Hodgdon et al. 2018). Thus, timed devices, releasing pheromone only at particular times of the day when adults are sexually active, could minimize the use of pheromone when the insects are not normally looking for mates. Moreover, because female cecidomyiids release very small quantities of pheromone (ca. pg) and male cecidomyiid antennae are acutely sensitive to minute amounts (Hall et al. 2012), pheromone-mediated mating disruption dispensers for midges could release much smaller, and thus cheaper, amounts of material compared with systems for pests in other insect orders.

Due to its structural complexity and chirality, the swede midge pheromone is costly to synthesize (Hillbur et al. 2005; Samietz et al. 2012), limiting its commercial feasibility. However, Samietz et al. (2012) demonstrated successful swede midge mating disruption using a stereospecific blend. Currently, the stereospecific blend is used primarily for monitoring and to inform insecticide spray programs in some crops (Hallett et al. 2007; Hallett and Sears 2013). The swede midge pheromone comprises a 1:2:0.02 ratio of (2*S*, 9*S*)-diacetoxyundecane, (2*S*, 10*S*)-diacetoxyundecane, and (*S*)-2-acetoxyundecane (Hillbur et al. 2005), respectively. Each component has one or two chiral centers, and therefore, multiple stereoisomers. Synthesis that results in production of other stereoisomers, rather than stereospecific synthesis of just the main compound (2*S*, 10*S*)-diacetoxyundecane, can reduce the cost of producing a pheromone blend (G. Lopez, Chemtica International, pers. comm.), but also results a loss of attraction to males (Boddum et al. 2009).

Unexpectedly, Boddum et al. (2010) found that males also possess receptors for at least one of the non-natural stereoisomers of 2, 10-diacetoxyundecane, although their behavioral function is unknown. It is possible that swede midge congeners produce other stereoisomers of 2, 10-diacetoxyundecane. For some insects, the ability to detect and avoid pheromone plumes from closely related species aids in recognition of con- and heterospecifics (Symonds and Elgar 2008). Although male swede midge are not attracted to pheromone blends containing non-natural stereoisomers of 2, 10-diacetoxyundecane, such blends may present a lower-cost alternative for pheromone-mediated mating disruption, for which pheromone attractiveness may not be necessary (Evenden et al. 1999; Miller and Gut 2015; Stelinski et al. 2008).

More economical racemic pheromone blends could be useful for pheromone-mediated mating disruption, although their efficacy for swede midge is, as yet, unknown. Racemic or non-natural stereoisomers of pheromone components have been successfully used for pheromone-mediated mating disruption of other important pest species, including gypsy moth, *Lymantria dispar*, (Lepidoptera: Lymantriidae) and the white grub beetle, *Dasylepida ishigakiensis*, (Coleoptera: Scarabaeidae), even though these blends are less attractive to males (Arakaki et al. 2013; Onufrieva et al. 2008). Although Boddum et al. (2009, 2010) investigated male swede midge attraction and antennal responses to racemic blends, additional research is necessary to determine whether such blends can disorient mate-seeking males.

Although the complete racemic blend is not attractive to male swede midges (Boddum et al. 2009), we hypothesized that it may be effective at disorienting males and preventing mate location in a mating disruption system. We tested behavioral responses of male swede midges exposed to three blends to determine candidate blends for mating disruption: the natural blend of all stereospecific compounds, the complete racemic blend containing all possible stereoisomers of each compound, and a mixed stereospecific/racemic blend, which contained (2*S*, 10*S*)-diacetoxyundecane, necessary to attract males, and racemic blends of the other two components (2, 9- diacetoxyundecane and 2-acetoxyundecane). Specifically, our research questions were: 1) how does pheromone dose affect male attraction, 2) which pheromone blend(s) do male midges prefer when given a choice, 3) which pheromone blends elicit male upwind flight and courtship behavior, and 4) which pheromone blends prevent males from locating and mating with females in a controlled laboratory setting? Our overall objective was to determine candidate pheromone blends for future mating disruption trials in the field.

Methods and Materials

Swede Midge Colony Rearing

We reared swede midges in a laboratory colony for our behavioral assays. The midge colony originated from the Swiss Federal Research Station for Horticulture in Wädenswil, Switzerland, and was previously reared at the University of Guelph in Ontario, Canada prior to importing the colony to our laboratory at the University of Vermont in Burlington, Vermont, USA (USDA APHIS permit number P526P-13-03136). To avoid genetic bottlenecking in the colony, we periodically added field-collected midges from Vermont, USA. The colony was kept at 22.4 ± 1.2 °C and 40.7 ± 11.4% RH

under a 16:8 light:dark photoperiod. We used *Brassica oleracea* group Botrytis 'Snow Crown' (cauliflower; Harris Seeds, Rochester, NY, USA) for rearing due to its suitability as a swede midge host (Hallett 2007). Plants received fertilizer at a rate of 150 ppm with two parts 21–5-20 and one part 15–0-14 with supplemental magnesium, and were grown in Fafard 3B soilless potting medium (Sun Gro Horticulture, Agawam, MA, USA). We introduced 6–8 week old plants into rearing cages for oviposition when the cauliflower buds were approximately 3 cm diam. After plants were exposed to adult midges for 24–72 h, we moved them to separate cages to allow larvae to develop. Once larvae reached third instar, after 14 d, we cut the stems of the cauliflower plants and inserted the buds into the potting media to facilitate movement of larvae into the media for pupation. When ready to pupate, larvae jump from or crawl down the stems of their host plants into the soil below (Readshaw 1961). We then returned the infested pots to the oviposition cages.

Test Insects

We used virgin midges less than 24 h old. Adult swede midges typically live for one to three days. Females mate only once, usually within the first day after eclosion (Readshaw 1961). We used a combination of individuals emerging from the laboratory colony and from isolated single female progenies. In our laboratory, most midges eclose shortly after dawn (Hodgdon et al. 2018). We captured individuals as they emerged from the soil and transferred males and females to separate containers to prevent mating prior to behavioral assays.

We also reared offspring cohorts from individual females in deli containers (Webstaurant Store, Lititz, PA, USA) to separate the emerging males and females. A majority of cecidomyiid females produce either only male or only female progeny (Benatti et al. 2010; Stuart and Hatchett 1991), which may be a strategy to prevent inbreeding (Tabadkani et al. 2011). To produce unmated offspring, we caged one female and two or three males from the main colony in a modified plastic deli container (two 946 ml containers fastened together), each with an 8–10 week old cauliflower plant. We cut the cauliflower meristems and inserted them partially into the soil after 14 d, similar to our colony rearing protocol. We aspirated adult offspring emerging in the containers ca. 18–21 days later singly into vials and held them in the experiment room for at least 30 min prior to our trials.

Swede Midge Pheromone

For all of our behavioral trials, we formulated blends so that the amounts of the naturally produced stereoisomers for each component were equal across stereospecific and racemic blends (Table 1), similar to those used by Boddum et al. (2009) in their wind tunnel studies with swede midge. We obtained >98% optically pure swede midge pheromone components from ChemTica Internacional (Santo Domingo, Heredia, Costa Rica) and formulated the following blends: solvent only (hexane) control, stereospecific blend containing each of the three naturally-produced stereoisomers, racemic blend containing all possible stereoisomers for each compound, and a stereospecific/racemic blend (Table 1). The stereospecific/racemic blend contained (2*S*, 10*S*)-diacetoxyundecane, required for male attraction, and the racemic blends of the other components (2, 9- diacetoxyundecane and 2- acetoxyundecane), for which the non-natural stereoisomers do not inhibit attraction (Boddum et al. 2009). Because each of 2, 9- and 2, 10-diacetoxyundecane has four stereoisomers (*RR, RS, SS*, *SR,* or *meso*-) and the *SS*- stereoisomer is only 25% of the total amount (Hillbur et al. 2005), the racemic mixture was tested at a dose four times higher than the *SS*- stereoisomer (Table 2). We needed only twice as much 2-acetoxyundecane, because this compound has only one chiral center (Hillbur et al. 2005). For each experiment, we delivered the pheromones in 10 μl solutions with HPLC-grade hexane (Fisher Scientific, NH, USA) onto VWR qualitative #413 white filter paper (VWR International, Radnor, PA, USA), similar to Hillbur et al. (2005). Our doses ranged greatly due to the different sizes and air volumes of the treated areas in the two devices: picograms for the y-tube olfactometer trials and nanograms for the wind tunnel experiment (Table 2).

Male Dose-Response to Natural (Stereospecific) Pheromone

To determine which doses to use in the subsequent y-tube olfactometer choice experiments, we conducted a sensitivity experiment to test male attraction to different doses of the stereospecific pheromone blend in a y-tube olfactometer. We recorded whether midges were attracted to and moved toward the pheromone source or the solvent-only control, or exhibited no upwind movement. Lack of insect movement can be an indicator of both excessively small and large pheromone doses (Farkas et al. 1974; Shorey 1973). Although it is unknown how much pheromone a single swede midge female produces ("female equivalent" doses), gland extracts from the congeneric *C. pisi* (pea midge) yielded only a few picograms (Hall et al. 2012). Our highest dose [4 ng of (2*S*, 10*S*)-diacetoxyundecane, 2 ng (2*S*, 9*S*)-diacetoxyundecane and 0.04 ng (*S*)-2-acetoxyundecane] was based upon estimates of female equivalents from Hessian fly (Y. Hillbur, pers. comm.). Using 4 ng of (2*S*, 10*S*)-diacetoxyundecane as a starting point, we tested whether males would respond to decreasing serial dilutions [0.4, 0.04, 0.004, and 0.0004 ng of (2*S*, 10*S*)-diacetoxyundecane, with (2*S*, 9*S*)-diacetoxyundecane and (*S*)-2-acetoxyundecane in a 1:0.02 ratio] to create a dose-response curve. We placed the pheromones into one arm of the y-tube. Our control treatment, in the other arm, consisted of 10 μl hexane.

Table 1 Stereoisomers of swede midge pheromone components in blends used in behavioral assays

Pheromone blend	Diacetoxyundecane				Acetoxyundecane	
	2*S*, 9*S*	2*R*, 9*R* 2*R*, 9*S* 2*S*, 9*R*	2*S*, 10*S*	2*R*, 10*R* *meso*	2*S*	2*R*
Stereospecific	X	–	X	–	X	–
Stereospecific/Racemic	X	X	X	–	X	X
Racemic	X	X	X	X	X	X
Control	–	–	–	–	–	–

The y-tube olfactometer (Sigma Scientific, Micanopy, FL) consisted of an air compressor delivering air through activated carbon filters, through flow meters, and into Teflon tubing. The tubing was attached to two 10 cm-long glass odor adapters, fitted onto both stems of a y-tube. Each odor adapter consisted of a glass tube with a tapered end fitted with metal screen to prevent insects from contacting the odor within the adapter. The inner diameter of the y-tube was 1.8 cm, the distance from the end of the stem to the junction 14.5 cm, and the arms 8 cm long. The olfactometer was set up in a separate room at 22.8. ± 0.54 °C. Because swede midges are small (~2 mm in length) and relatively weak fliers in a y-tube (pers. obs.), we set the airflow through each arm of the y-tube at 0.3 $l.min^{-1}$.

We tested male responses to pheromone within 3 hrs after the onset of photophase, when female midges typically release pheromone (Hodgdon et al. 2018). Within the y-tube, each male had five min. to respond to the pheromone or control stimulus. If a midge traveled >2.5 cm past the y-tube junction and remained there for at least 15 s., we recorded a positive response. When midges did not make a choice within the time limit, they were removed and not tested again. To remove directional bias, we flipped the y-tube arms 180° after each replicate, and randomly selected a different concentration to test every five midges. We tested the treatments in random order, with five males comprising one block, and a total of eight blocks, for a total of $n = 40$ replicate midges for each treatment. Between each block, we cleaned glassware with hexane and allowed the pieces to dry in air. We recorded male responses with a binary scoring system: flight toward the pheromone (1), or either no flight or flight toward the solvent-only control (0).

To test whether the number of midges flying toward pheromone differed significantly from 50%, we used a series of binary exact tests for each pheromone dose. Because we used the same pheromone source (filter paper) for five midges within treatment groups, we first conducted chi-squared tests to determine if the pheromone source (individual filter paper) influenced the distribution of midge responses within each pheromone treatment. Because we found that midge responses to specific pheromone sources (filter papers) did not differ within all of our pairwise comparisons ($P > 0.05$), we did not include pheromone source as a variable in our final analyses. For all statistical analyses, we used SPSS statistical software version 22 (International Business Machines, Armonk, NY, USA). We interpreted statistical significance of our results using $\alpha = 0.05$ and Bonferroni-corrected P values where necessary for multiple comparisons.

Table 2 Pheromone treatments used for swede midge pheromone attraction and preference experiments in the y-tube olfactometer and wind tunnel

Pheromone blend	Diacetoxyundecane				Acetoxyundecane	
	2*S*, 9*S*	2,9	2*S*, 10*S*	2,10	2*S*	2
Y-tube olfactometer (control *vs.* pheromone)						
Stereospecific	2 pg	–	4 pg	–	0.04 pg	–
Stereospecific/Racemic	–	8 pg	4 pg	–	–	0.08 pg
Racemic	–	8 pg	–	16 pg	–	0.08 pg
Control	–	–	–	–	–	–
Y-tube olfactometer (pheromone *vs.* pheromone)						
Stereospecific	1 pg	–	2 pg	–	0.02 pg	–
Stereospecific/Racemic	–	4 pg	2 pg	–	–	0.04 pg
Racemic	–	4 pg	–	8 pg	–	0.04 pg
Control	–	–	–	–	–	–
Wind tunnel						
Stereospecific	10 ng	–	20 ng	–	0.2 ng	–
Stereospecific/Racemic	–	40 ng	20 ng	–	–	0.4 ng
Racemic	–	40 ng	–	80 ng	–	0.4 ng
Control	–	–	–	–	–	–

Pheromone Choice

We tested if midges preferred one pheromone blend to another (Table 1) using a series of six pairwise comparisons in a y-tube olfactometer. Three of the comparisons consisted of a control (hexane only) versus the stereospecific, stereospecific/racemic, or racemic pheromone blends, and the remaining comparisons consisted of one pheromone blend versus another (stereospecific versus stereospecific/racemic, stereospecific versus racemic, and stereospecific/racemic versus racemic). For comparisons of a pheromone blend versus the control, we used the most attractive dose of the pheromone blend (4 pg; Fig. 1; Table 2). For the remaining comparisons comparing different pheromone blends, the total amount of pheromone delivered to males in each arm was equal to half the dosage used in pheromone-control setups, so that the total amount delivered to males was equal to the most attractive amount.

We used unmated males for the experiments, using the same olfactometer protocol as described for the sensitivity assays, for a total of $n = 70$ replicate midges for each comparison. Unlike the dose-response experiments, we excluded males that did not make a choice from further analysis, based on the protocol used by Andersson et al. (2009), and because we previously determined that our dosages were appropriate based on the sensitivity experiments. Unresponsive midges may have had differing pheromone sensitivity and/or differing circadian patterns of sexual activity, or may have been harmed during handling. We used binomial exact tests to examine differences between the proportions of midges choosing one pheromone treatment over another in each of our y-tube setups, similar to our dose-response analyses.

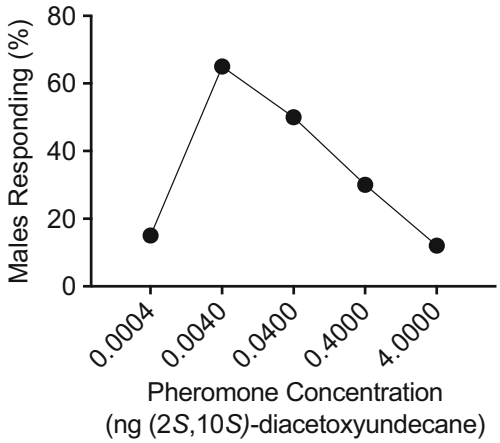

Fig. 1 Percentage of male swede midges ($n = 40$) flying to the pheromone source in a y-tube olfactometer. Pheromone concentrations are displayed in amounts of (2*S*, 10*S*)-diacetoxyundecane, the main component in the swede midge pheromone blend, with (2*S*, 9*S*)-diacetoxyundecane and (*S*)-2-acetoxyundecane following in the ratio produced by females (2:1:0.02, respectively) (Hillbur et al. 2005). Connecting lines offer visual guidance between data points rather than interconnectedness of data. The 0.0040 ng dose of (2*S*, 10*S*)-diacetoxyundecane was the only treatment that attracted numbers of midges greater than 50% ($P = 0.017$)

Male Flight and Courtship Behavior in Response to Pheromone Blends

We tested whether male midges exhibited upwind movement and courtship behavior in response to single pheromone blends in a wind tunnel, similar to those used by Hillbur et al. (2005) and Boddum et al. (2009) for swede midges. Swede midge courtship behavior is similar to other plant-feeding midges and lepidopterans. After detecting pheromone, male midges fly upwind in a zigzag pattern toward a female or pheromone source (Boddum et al. 2009; Gagne 1989; Hillbur et al. 2005). When males get close to the pheromone source or female, they fan (vibrate) their wings. When a pheromone signal is lost, adulterated, or unattractive, male insects may cease to travel farther upwind (Shorey 1973). Therefore, we assumed that the farther a midge traveled upwind within the tunnel, the more it was attracted to a treatment.

The tunnel (50 × 50 × 170 cm) consisted of an acrylic structure with activated carbon filters and mesh screens on both ends to remove contaminants and smooth the flow. We used a household box fan (51 × 51 cm) to push air through the tunnel. We confirmed that the filter slowed the air 0.5 $cm.s^{-1}$ using a hot wire anemometer (model 55P16, Dantek Dynamic, Skovlunde, Denmark). Using smoke from a smoke pen placed at the upwind end, we confirmed that the airflow through the wind tunnel was reasonably smooth. We placed filter papers with pheromone into a bent wire paper clip holder on an overturned glass beaker at the upwind end of the tunnel. To minimize contamination, we set up the tunnel in a room free of plants and insects. The building's ventilation system exchanged air in the room ca. every 8 min. and was on average 26.7 ± 1.1 °C. The tunnel was illuminated by sunlight through windows as well as by 40 W fluorescent lights hung above and parallel to the tunnel. We used a handheld light meter (Enviro-Meter, Control Company, Webster, TX, USA) to adjust the setup so that light level was approximately equal at both ends of the tunnel.

Using a randomized complete block design, we observed the responses of $n = 50$ males to the four pheromone treatments, using the same doses as Boddum et al. (2009; Table 2). To avoid contamination between pheromone treatments and airborne pheromone buildup in the experiment room, we tested 10 males to only one pheromone treatment per day. We conducted the experiments between 2 and 4 h after the onset of photophase, the peak hours of male mate-searching activity (Hodgdon et al. 2018). After releasing single males from glass vials into the tunnel 120 cm away from the platform, we gave each male three min. to respond. We chose a 3 min. timeframe because pheromones evaporate

quickly from filter paper (Y. Hillbur, pers. comm.), and because midges typically responded within the first one or two min. We categorized male attraction by recording whether males exhibited the following behaviors: wing fanning, flight and/or landing at least halfway to the pheromone source (60 cm), flight and/or landing within 5 cm of the pheromone source, and flight and/or landing on the filter paper. We replaced the filter paper every 3 min. or a maximum of two replicates. We cleaned the tunnel with 70% ethanol and allowed the fan to push clean air through the tunnel after each block for at least 1 hr to remove residual pheromone. We used chi-square analyses of wing fanning, flying halfway, within 5 cm, and making contact with the pheromone source to determine whether males responded to the pheromone treatments differently.

Simulated Pheromone-Mediated Mating Disruption

Using the y-tube olfactometer, we created a simulated pheromone-mediated mating disruption system (Fig. 2) to test whether male midges could locate and copulate with intermittently calling females against a background of synthetic pheromone. Both arms contained a filter paper loaded with equal doses of the same pheromone blend (Table 3). In the first arm, beyond the wire screen separating the filter paper in the odor adapter and the y-tube, we placed five unmated females with access to the male. The second arm contained no insects. We gave males ten min. to mate with the females after being released into the y-tube.

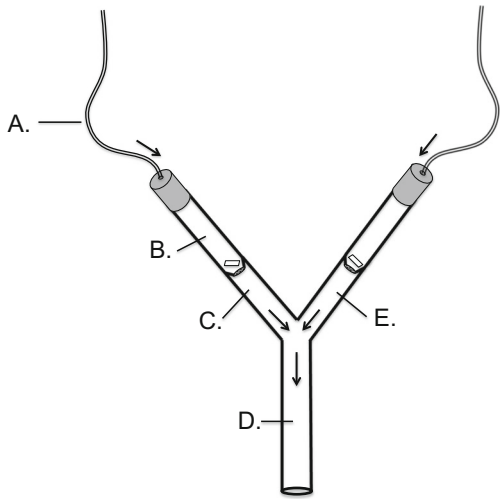

Fig. 2 Y-tube for swede midge pheromone-mediated mating disruption experiment (not to scale). Air entered each arm of the y-tube via Teflon tubing (**a**) from an air compressor and carbon filters (not shown). Air passed through odor adaptors (**b**) containing filter paper with pheromone treatments (Table 2), in which each arm received the same pheromone treatment. Five live females loaded into one arm (**c**) were exposed to the male released into the stem of the y-tube (**d**). The remaining arm (**e**) contained no females

We used pheromone doses tenfold higher than the dose most attractive to midges in the olfactometer dose-response experiment (Tables 2 and 3, Fig. 1), and loaded both arms with equal doses of the same pheromone blend on filter paper. This was the lowest dose at which midges in our sensitivity experiment demonstrated behaviors consistent with arrestment (Fig. 1). Arrestment, when males reduce mate-searching behavior in the presence of high ambient levels of pheromone, due to sensory impairment or other factors, is one mechanism in which mating disruption prevents mate location (Miller and Gut 2015). Our picogram pheromone doses were exponentially ($1 \times e^{-6}$) lower than Samietz et al.'s (2012) microgram loading rates for swede midge mating disruption dispensers for agricultural field use [50 μg (2*S*, 9*S*)-diacetoxyundecane, 100 μg (2*S*, 10*S*)-diacetoxyundecane and 1 μg (*S*)-2-acetoxyundecane], due to the exponentially smaller volume of air in our y-tube compared with open-air field plots.

We conducted the mating disruption simulation experiment in the morning (within 3 h following the onset of photophase) and in the evening (4 h prior to scotophase), which is when males and females display mate-seeking behavior (Hodgdon et al. 2018). We used a binary scoring system to record whether or not males for each pheromone treatment ($n = 32$) copulated with at least one female. As described by Readshaw (1961), we recorded copulation when we observed the abdomens joined for at least 5 s. In between each replicate, we cleaned glassware with hexane and replaced pheromone sources to avoid contamination among treatments. We tested the pheromone treatments using a randomized complete block design, with one replicate per treatment per block.

We tested how pheromone blend, time of observation (morning or evening), and the interaction between the two influenced the probability of copulation using a binary logistic regression model. Because both time and the interaction term were not significantly associated with copulation, we pooled data from our morning and evening observations together for the final model. We used a series of chi square tests to evaluate pairwise comparisons between pheromone treatments.

Results

Male Dose-Response to Natural (Stereospecific) Pheromone

We found that pheromone concentrations varied in attractiveness to male midges (Fig. 1). The 0.004 ng dose of (2*S*, 10*S*)-diacetoxyundecane (with other two components) was the only dose that attracted more than 50% of midges ($P = 0.017$). The highest dose, 4 ng, was the least attractive, attracting only 12.5% of midges. Midges that did not fly toward the pheromone treatments either avoided pheromone by entering the

Table 3 Pheromone treatments in each y-tube arm used in swede midge simulated pheromone-mediated mating disruption experiment

Pheromone blend	Diacetoxyundecane				Acetoxyundecane	
	2*S*, 9*S*	2, 9	2*S*, 10*S*	2, 10	2*S*	2
Stereospecific	20 pg	–	40 pg	–	0.4 pg	–
Stereospecific/Racemic	–	80 pg	40 pg	–	–	0.8 pg
Racemic	–	80 pg	–	160 pg	–	0.8 pg
Control	–	–	–	–	–	–

control arm of the y-tube, or exhibited arrestment by remaining stationary within the release point of the stem.

Pheromone Choice

When given a choice between two blends in a y-tube, males were ca. three times more likely to prefer the stereospecific and stereospecific/racemic pheromone blends compared to the control and racemic blends ($P < 0.05$ for each comparison; Fig. 3). Males did not exhibit a preference between the stereospecific and stereospecific/racemic blends, and the difference between the numbers of males choosing the stereospecific pheromone blend over the stereospecific/racemic mixture was not significant ($P > 0.05$). When given a choice between the racemic and control treatments, four times more males ($P < 0.001$; 79%) chose the control over the racemic treatment. Males behaved as though repelled by the racemic blend, flying into the only location within the y-tube not permeated with the compounds, the arm containing the solvent. Across all treatments, on average one in ten midges did not make a choice in the y-tube and were excluded from the analyses.

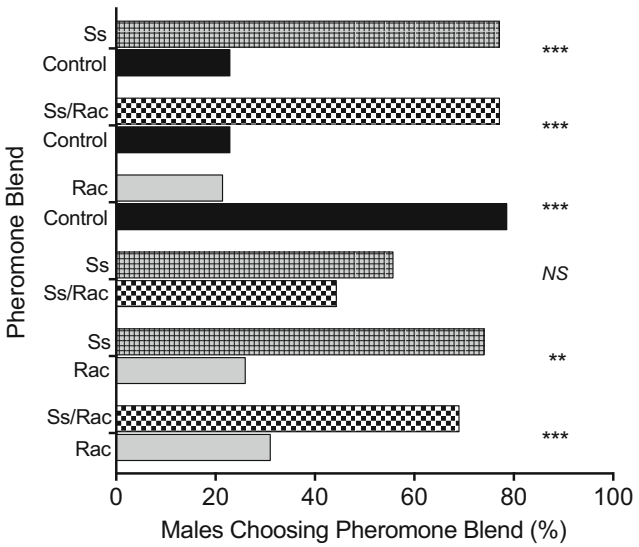

Fig. 3 Percentage of male swede midges ($n = 70$ each treatment) choosing pheromone blends (Table 2; where Ss = stereospecific and Rac = racemic) when given a choice between two blends in the y-tube olfactometer. *NS*, **, and *** indicate non-significance ($P > 0.05$), and statistical significance at $P < 0.01$ and $P < 0.001$, respectively, for individual pairwise pheromone comparisons

Male Flight and Courtship Behavior in Response to Pheromone Blends

Males were more attracted to the stereospecific and stereospecific/racemic pheromone treatments, as few to no males flew toward the control or racemic treatments. The stereospecific blend attracted the most males landing within 5 cm of the pheromone source (66%, Fig. 4), compared to all other pheromone treatments ($P < 0.05$), except for the stereospecific/racemic blend ($P > 0.05$). Males were 16 times more likely to fly within 5 cm of the stereospecific blend versus the racemic blend. Percentages of midges flying within 5 cm of the racemic (4%) and control (0%) treatments did not differ ($P > 0.05$). Few males flew halfway (60 cm) when exposed to these treatments (4% and 0%, respectively), and most exhibited no upwind flight. When exposed to the racemic blend, more than half of males (55%) appeared to be repelled, reversing flight direction in the tunnel and landing on the back wall at the farthest point from the pheromone source.

The pheromone blends also differed in terms of eliciting wing fanning, a male courtship behavior exhibited following landing near females or pheromone. The likelihood of wing fanning varied across the treatments ($\chi^2_3 = 57.43$, $P < 0.001$). We observed the highest percentage of males (54%) fanning wings in response to the stereospecific treatment (Fig. 5). Males were almost three times as likely to fan their wings when exposed to the stereospecific blend versus the stereospecific/racemic blend. Few (2% or 0%) males fanned wings in response to the racemic or control treatments, respectively, indicating that they were not stimulated by these blends.

Simulated Pheromone-Mediated Mating Disruption

In our simulated pheromone-mediated mating disruption system, all pheromone blend treatments reduced the ability of males to locate and copulate with calling females ($\chi^2_3 = 38.017$, $P < 0.001$). Out of $n = 32$ replicate midges for each treatment, only one male was able to copulate with a female in each of the stereospecific and racemic treatment groups. No males copulated in the presence of the stereospecific/racemic treatment, whereas 14 males copulated in the control treatment (Fig. 6). On average, 18% of males across all treatments exhibited arrestment (failure to leave the stem of the y-tube)

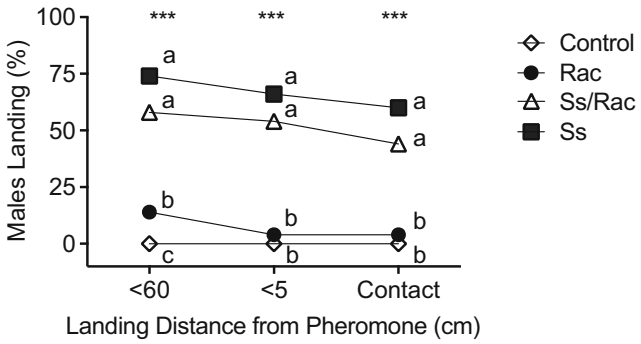

Fig. 4 Percentage of male swede midges ($n = 50$) landing within 60 cm, 5 cm, and making contact with the pheromone source (Table 2; where Ss = stereospecific and Rac = racemic) in the wind tunnel. Connecting lines offer visual guidance between data points rather than interconnectedness of data. Data points with the same letter at the same landing distance are not different based on chi square post hoc tests ($P > 0.05$); *** indicates significance of the overall model at each distance at $P < 0.001$

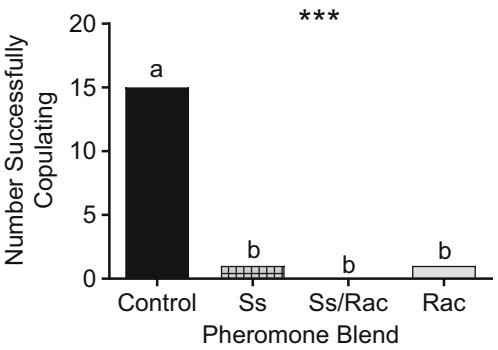

Fig. 6 Observed incidences of swede midge copulation ($n = 32$ replicate males for each treatment) in the y-tube olfactometer in the presence of different pheromone blends (Table 3; where Ss = stereospecific and Rac = racemic), in the simulated pheromone mating disruption experiment. Treatments with the same letter are not different ($P > 0.05$) based on post hoc chi square tests; *** indicates significance of the overall model at $P < 0.001$

versus 0% of control males; the remaining males entered the y-tube arms but did not copulate with females.

Discussion

Despite the widely held belief that the most effective pheromone blends for mating disruption are the most attractive ones, less attractive blends may also disorient males (Arakaki et al. 2013; Evenden et al. 1999; Miller and Gut 2015; Thorpe et al. 1999). We argue that less attractive blends should be considered for mating disruption systems if they are more economical and function as well as natural blends. In our simulated pheromone-mediated mating disruption setup, we found that unattractive racemic swede midge pheromone blends functioned similarly to attractive stereospecific blends, by preventing males from copulating with females. Given that the racemic blend is less expensive to produce than the stereospecific blend, it may prove to be useful for mating disruption systems.

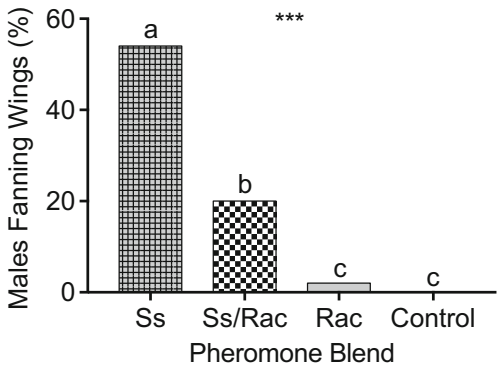

Fig. 5 Percentage of male swede midges ($n = 50$ for each treatment) fanning wings after landing in response to pheromone blends (Table 2; where Ss = stereospecific and Rac = racemic) in a wind tunnel. Treatments with the same letter are not different ($P > 0.05$) based on post hoc chi square tests; *** indicates statistical significance of the overall model at $P < 0.001$

Males responded differently to the two pheromone blends containing racemic compounds. One of the blends, stereospecific/racemic, contained non-natural stereoisomers of 2, 9-diacetoxyundecane and 2-acetoxyundecane along with the natural stereoisomer of the main pheromone component, (2*S*, 10*S*)-diacetoxyundecane. This blend functioned similarly to the complete stereospecific blend in eliciting male flight, as males do not possess receptors for any of the non-natural stereoisomers of 2, 9-diacetoxyundecane and 2-acetoxyundecane (Boddum et al. 2010). However, we observed more wing fanning in response to the stereospecific blend versus the stereospecific/racemic blend in the wind tunnel, indicating that males were more stimulated by the natural blend, at least near the source. When we used racemic blends of all three compounds, midges were repelled. Because we did not observe a difference in male attraction to the stereospecific or stereospecific/racemic blends, the stereospecific/racemic blend may be a lower cost alternative to the stereospecific blend, facilitating more affordable monitoring lures and pheromone-mediated mating disruption systems.

Both racemic blends appeared to be equally effective in preventing copulation in our y-tube setup. Only one male was able to locate and mate with females in each of the stereospecific and racemic treatments. Many males exposed to the pheromone treatments exhibited arrestment, remaining in the stem of the y-tube and not searching for mates in our simulated mating disruption setup. These males may have been over-stimulated or desensitized by attractive pheromones, not attracted by a particular blend, or unresponsive to pheromone due to differing circadian rhythms compared to the majority of the population.

Non-natural pheromone blends can elicit a range of behaviors that can contribute to mating disruption. For example,

some pheromone blends target multiple receptors in the antennae and could potentially elicit more behavioral effects to prevent mating than others. Miller and Gut (2015) argued that pheromone blends causing multiple behavioral impairments are necessary for successful mating disruption. For example, some synthetic pheromones desensitize male sensory systems and prevent normal response to calling females, including arrestment, sensory impairment and/or habituation (Cardé and Minks 1995; Daly and Figueredo 2000; Judd et al. 2005; Stelinski et al. 2008). Synthetic blends can also mix with female pheromones, adulterating the chemical composition of the female plume and decreasing male attraction (Miller and Gut 2015). Ultimately, all of these behaviors may reduce mate location success, and their effects may be enhanced when multiple mechanisms operate simultaneously.

Our results indicate two promising attributes of swede midge pheromone biology that could be exploited for more economical pheromone-mediated mating disruption on the field: 1) midges are responsive to minute pheromone amounts, indicating little pheromone material is needed to disorient males, and 2) certain blends with lower-cost racemic compounds were equally effective in disorienting mate-seeking males as natural stereoisomeric blends. Because there are currently no effective insecticides for swede midge management approved for organic production (Evans and Hallett 2016; Seaman et al. 2014), pheromone-mediated mating disruption may be viable as an alternative to insecticides for managing this pest in organic cropping systems. Future research testing racemic pheromone components in field mating disruption is a practical next step in the development of this pest management tactic for swede midge.

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