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DOI: 10.1111/eea.12560

Rowley, C., Pope, T.W., Cherrill, A., Leather, S.R., Fernandez-Grandon, G.M. and Hall, D.R. 2017. Development and optimisation of a sex pheromone lure for monitoring populations of saddle gall midge, Haplodiplosis marginata. *Entomologia Experimentalis et Applicata.*

30 March 2017

Development and optimisation of a sex pheromone lure for monitoring populations of saddle gall midge, *Haplodiplosis marginata*

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Key words: (*R*)-2-nonyl butyrate, chirality, wheat, traps, dispensers, electroantennography

Running title: *Haplodiplosis marginata* pheromone trap development

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Abstract

Saddle gall midge, *Haplodiplosis marginata* (von Roser) (Diptera: Cecidomyiidae), is a sporadic pest of cereals in Northern and Central Europe and is of increasing importance in the UK. Recently the major component of the sex pheromone produced by adult female *H. marginata* was reported to be 2-nonyl butyrate. The importance of absolute configuration on attractiveness, the effects on trap catches of the addition of minor pheromone components, dispenser type and pheromone loading are described in the development of an optimised pheromone lure with which to trap *H. marginata* males. In analyses of volatiles collected from virgin female *H. marginata* by gas chromatography (GC) coupled to electroantennographic recording (EAG) from the antenna of a male *H. marginata*, two EAG responses were observed. Analyses by coupled GC-mass spectrometry (MS) indicated these were due to 2-nonyl butyrate and a trace amount (1%) of 2-heptyl butyrate. A similar trace amount of 2-nonanol was detected in GC-MS analyses but this compound did not elicit an EAG response when the synthetic compound was tested while the other two compounds did. These three compounds were not observed in collections of volatiles made from male *H. marginata*. The 2-nonyl butyrate was shown to be the (*R*)-enantiomer, and in field trapping tests (*R*)-2-nonyl butyrate was at least ten times more attractive to male *H. marginata* than the racemic compound while the (*S*)-enantiomer was unattractive. Addition of the potential minor components individually or together at the naturally-occurring ratios did not increase or reduce the attractiveness of the lure. Polyethylene vials and rubber septa were equally effective as pheromone dispensers, lasting for at least five weeks in the field in the UK, although laboratory tests indicated release from the former was more uniform and more likely to last longer in the field. Increasing loading of pheromone in the dispenser increased attractiveness. Traps baited with polyethylene vials containing 0.5 mg of (*R*)-2-nonyl butyrate are recommended for monitoring *H. marginata* and these are far more sensitive than water or sticky traps currently used for monitoring this pest.

Introduction

Saddle gall midge, *Haplodiplosis marginata* (von Roser), is a sporadic pest of cereals of increasing importance in the UK and parts of continental Europe. Yield losses of up to 70% have been reported during recent UK outbreaks (Ellis et al., 2014). It is a univoltine species with a short-lived adult stage and an overwintering phase in the larval stage. Adults begin emerging around the start of May (Skuhravý et al., 1983; Gratwick, 1992) and mating occurs immediately (Golightly & Woodville, 1974). Females lay their eggs on the leaves of cereals and other grasses and, once hatched, the larvae begin feeding on the stem of the host plant resulting in the formation of saddle-shaped galls beneath the leaf sheath (Skuhravý et al., 1983; Dewar, 2012). Gall formation can damage the plant by restricting nutrient flow to the ear leading to under-filled grains, and by leaving the plant vulnerable to attack from secondary pathogens (Nijveldt & Hulshoff, 1968; Dewar, 2012). Severe infestation results in multiple galls along the stems of a cereal plant, weakening the stems and increasing the risk of stem breakage which can cause substantial yield loss (Gratwick, 1992; Berry et al., 1998). Crops most at risk from *H. marginata* are spring wheat and barley and late-sown winter wheat, particularly in areas with heavy soils (Golightly & Woodville, 1974; Skuhravý et al., 1983, Skuhravý et al., 1993; Pope & Ellis, 2013).

The pest is very sporadic, and the exact conditions which influence diapause termination, emergence and the reproductive success of *H. marginata* are unknown, making outbreaks difficult to forecast (Woodville, 1973; Basedow, 1986). As in other Cecidomyiidae, monitoring populations of *H. marginata* is difficult due its cryptic nature (Harris & Foster, 1999). Additionally the timing of pesticide application is critical as once the larvae begin feeding they are protected from contact insecticides by the leaf sheath. The best control is achieved when sprays coincide with the first appearance of adults or eggs in the crop, or 7 – 10 days after the start of adult emergence (Ellis et al., 2014). Consequently, there is an urgent need for a simple and effective monitoring system for growers to use and on which pest management decisions can be based (Censier et al. 2015, 2016b).

Monitoring of pest populations allows insecticides to be applied judiciously to target the temporal occurrence of the vulnerable life-stage of the organism (Jones, 1998). Use of pheromone-baited traps has been found to be an effective method of population monitoring in many pest species (Hardie & Minks, 1999; Witzgall et al., 2010), including a related cecidomyiid species, the orange wheat blossom midge, *Sitodiplosis mosellana* (Gehin) (Bruce et al., 2007; Bruce & Smart, 2009; Oakley & Ellis, 2009).

The major component of the *H. marginata* sex pheromone was recently identified as 2-nonyl butyrate and the synthetic racemic compound was found to be attractive to male insects in field trials in Belgium (Censier et al., 2014, 2016a). In our work we confirmed the basic findings of Censier et al. (2014) and extended them by using electroantennography to detect potential minor pheromone components. The effects of these were evaluated in field trapping tests, as was the importance of the absolute configuration of the major pheromone component and the effect of pheromone loading on trap catches. Practical pheromone dispensers were evaluated to provide farmers and agronomists with an effective monitoring system on which to base pest management decisions.

Materials and methods

Insects

Larvae of *H. marginata* were collected from soil samples taken from affected fields between November 2013 and May 2014 and stored at 4 °C for a minimum of three months. Each larva was transferred to an individual plastic container (1.5 cm diameter x 2.5 cm) of moist sterilised compost covered with a fine mesh and maintained at 20 °C, 60% RH, and a photoperiod of 16:8 (L:D) h until adults emerged.

Collection of pheromone

Volatiles were collected from individual virgin adult males and females separately, within 48 h of emergence. A single live midge was used per collection and was placed in a cylindrical

glass vessel (5.3 cm diameter x 13 cm; Hamilton Laboratory Glass Ltd, Margate, UK) with a glass frit and activated charcoal filter at one end (20 cm x 2 cm; 10-18 mesh, Fisher Chemicals, UK) and a collection filter at the other. The collection filter consisted of a Pasteur pipette (4 mm i.d.) containing Porapak Q (200 mg; 80-100 µm, Waters Associates Inc., USA) positioned between two glass wool plugs (Field Instruments Co. Ltd, Twickenham, UK). Air was drawn through the charcoal filter into the vessel containing the midge and out through the collection filter using a vacuum pump (M361C, Charles Austen Pump Ltd, UK) at a rate of 0.5 L/min. Collections were made continuously for a period of 48 h. Five collections were made from males and four from females. Volatiles were desorbed from the collection filters with dichloromethane (1.5 ml), concentrated under a stream of nitrogen and refrigerated prior to analysis.

Analysis by coupled gas chromatography-mass spectrometry (GC-MS)

Aliquots of volatile collections were analysed by coupled gas chromatography-mass spectrometry (GC-MS) using a Varian 3500 GC coupled to a Saturn 2200 MS (Agilent Technologies, Stockport, Cheshire, UK) operated in electron impact mode. A polar or non-polar GC column was used (30 m x 0.25 mm i.d. x 0.25 µ film thickness) coated with DBWax (Supelco, Gillingham, Dorset, UK) or VF5 (Agilent) respectively, and the oven temperature was held at 40°C for 2 min and then programmed at 10°C/min to 240°C. Compounds were identified by their mass spectra, their GC retention indices relative to the retention times of *n*-alkanes and comparison of retention indices and mass spectra with those of authentic synthetic standards.

Analysis by coupled gas chromatography-electroanntenography (GC-EAG)

Electroanntenography (EAG) coupled with gas chromatography (GC) was used to measure antennal responses of male and female *H. marginata* to collections of volatiles from females. An Agilent 6890N GC (Agilent) was used with fused silica capillary columns (30 m x 0.32 mm i.d. x 0.25 µ film thickness) coated with polar DB Wax (Agilent) and non-polar SPB1 (Supelco). Injections were splitless (220˚C) for the polar column and with programmed

temperature vaporising injector (held at 50°C for 0.2 min and then programmed at 600°C/min to 220°C) for the non-polar column. The carrier gas was helium (2.4 ml/min) and the oven temperature was held at 50˚C for 2 min and then programmed at 10˚C/min to 250˚C. The ends of the GC columns went into a push-fit Y-connecter that lead through a second Y-connector fitted with two equal lengths of deactivated fused silica capillary going to the flame ionisation detector (FID) and a glass T-piece, splitting the GC effluent 50:50. The effluent was collected in the T-piece for 17 sec before being blown over the antennal preparation for 3 seconds in a stream of air (200 ml/min) (Cork et al., 1990).

The antennae were prepared by excising the head from a live specimen, then removing one of the antennae and the tip of the remaining antenna using a sharp microscalpel. Antennal responses were recorded using a Syntech INR-2 micromanipulator assembly (Syntech, Hilversum, The Netherlands). Two newly-pulled glass capillary electrodes were filled with an electrolyte solution of 0.1M KCl with 1% polyvinylpyrrolidine (BDH Chemicals Ltd, UK) added to prevent evaporation. These were attached to silver wire electrodes mounted in micromanipulators. The insect preparation was mounted between the two glass electrodes with the head in the reference electrode and the distal end of the antenna in the recording electrode. The antennal responses were amplified x 10 and converted to digital format through the second detector channel of the GC. Data from FID and EAG were captured and processed with EZChrom Elite v 3.3.1 software (Agilent).

Analysis by enantioselective gas chromatography

Enantoselective gas chromatography was carried out on a CP-Chirasil-Dex CB column (25 m x 0.32 mm; 0.25 µm film thickness; Varian/Agilent) with helium carrier gas (2.4 ml/min), split injection (220°C; 20:1) and FID (220°C). The oven temperature was held at 60°C for 2 min and then programmed at 5°C to 200°C.

Chemicals

Unless otherwise stated, all chemicals were obtained from SigmaAldrich (Gillingham, Dorset, UK) and were at least 98% pure.

Racemic 2-nonyl butyrate was prepared by esterification of 2-nonanol with butyric acid in the presence of N,N'-dicyclohexylcarbodiimide (DCCD) and 4-dimethylamino-pyridine (DMAP) in dichloromethane (Neises & Steglich, 1978). The product was obtained in 93% yield after purification by flash chromatography on silica gel eluted with 2% diethyl ether in hexane and kugelrohr distillation (70°C/0.03 mm Hg). ^{1}H and ^{13}C nuclear magnetic resonance (NMR), infra red (IR) and mass spectral (MS) data were in agreement with those reported by Censier et al. (2014).

Racemic 2-nonyl butyrate was resolved into the two enantiomers by stirring with a catalytic amount of lipase acrylic resin from *Candida antarctica* in phosphate buffer (1 M K2HPO4) for 6 h with monitoring by enantioselective GC, which selectively hydrolysed the (*R*)-enantiomer (Hall et al., 2012) The product was chromatographed on silica gel eluted successively with 2%, 5%, 10%, 20% and 50% diethyl ether in hexane to give (*S*)-2-nonyl butyrate (98.7% enantiomeric excess by enantioselective GC) and (*R*)-2-nonanol. The latter was esterified as above to give (*R*)-2-nonyl butyrate (98.9% e.e.).

Racemic 2-heptyl butyrate was prepared similarly from 2-heptanol. This was resolved into the enantiomers with lipase from *Candida antarctica* to give the (*S*)- (97.8% e.e.) and (*R*)-enantiomers (98.2% e.e.).

Pheromone dispensers

Two different dispenser types were tested: polyethylene vials (26 mm x 8 mm x 1.5 mm thick, Just Plastics Ltd., London, UK) and white rubber septa (20 mm x 10 mm; International Pheromone Systems Ltd., The Wirral, UK). These were loaded with the pheromone dissolved in hexane (100 µl) and the solvent was allowed to evaporate.

Release rates were measured for dispensers loaded with 2-nonyl butyrate (1 mg) and maintained in a laboratory wind tunnel (27°C, 2.2 m/sec wind speed). Duplicate samples were removed at weekly intervals and the remaining pheromone extracted individually in hexane (5 ml) containing dodecyl acetate (1 mg) as internal standard. Extracts were analysed by GC with FID on a capillary column (30 m x 0.32 mm i.d. x 0.125 µ film

177 thickness) coated with DB5 (Agilent) with splitless injection (220°C) and the oven
178 temperature held at 50°C for 2 min and then programmed at 10°C/min to 250°C.
179 The amount of pheromone remaining in lures returned from field trapping tests was
180 measured similarly.

181 **Field trapping experiments**

182 Field trapping experiments were all carried out at sites with known soil populations of
183 *H. marginata*. Five experiments were performed. Experiments 1, 2, 3 and 4 were carried
184 out in Oxfordshire, UK (51°55"N, 1°10"W). Experiment 5 was carried out in
185 Buckinghamshire, UK (51°37"N, 0°48"W). All fields were in winter wheat and the
186 experiments were conducted during part of the flight season of *H. marginata,* coinciding with
187 wheat growth stages 39-59 (Zadoks et al., 1974).
188 For each experiment, pheromone dispensers were placed in standard red delta traps
189 (Agralan, Wiltshire, UK) containing a removable sticky insert (15 cm x 15 cm). Polyethylene
190 vials were used as dispensers for all experiments with the exception of Experiment 1. Traps
191 were hung from fibreglass canes and positioned at the height of the wheat ear. For
192 experiments 1-4, traps were laid out in a randomised complete block design with 10 m
193 between traps and 50 m between blocks. Adult *H. marginata* were identified based on
194 antennal and genital morphology (Harris, 1966) and counted using a bifocal microscope.
195
196 *Experiment 1 – pheromone dispensers.* Catches of male *H. marginata* in traps baited with
197 2-nonyl butyrate (1mg) formulated in the two different types of pheromone dispenser, rubber
198 septa and polyethylene vials, were compared with catches in an unbaited trap. Traps were
199 laid out in 4 replicated blocks and were in place between 15 May - 19 June 2014 and the
200 sticky inserts of the traps were changed after 6 days, at which time the treatments were re-
201 randomised within the blocks.
202

Experiment 2 - pheromone chirality. Catches in traps baited with lures containing (*R*)-2-nonyl butyrate (0.5 mg), (*S*)-2-nonyl butyrate (0.5 mg), the racemic mixture (1 mg) and an unbaited trap as control were compared. Traps were laid out in 4 replicated blocks and were in place between 5-19 June 2014.

Experiment 3 – effect of minor components. The effects of addition of two minor components to (*R*)-2-nonyl butyrate (0.5 mg) were tested. (*R*)-2-Nonanol or (*R*)-2-heptyl butyrate, each at 2% of the major component, were added to (*R*)-2-nonyl butyrate separately and in combination. These treatments were compared with lures containing (*R*)-2-nonyl butyrate (0.5 mg), lures containing the racemic mixture (1 mg), and with an unbaited trap as control. Traps were laid out in 10 replicated blocks and were in place between 18-29 May 2015. The sticky inserts of the traps were changed on days 4 and 9 of the experiment, with the treatments re-randomised within the blocks after each change.

Experiment 4 – pheromone loading. Trap catches with lures containing loadings of 2.5 mg, 0.5 mg, 0.05 mg and 0.005 mg of the major pheromone component, (*R*)-2-nonyl butyrate, were compared. Traps were laid out in 10 replicated blocks and were in place between 2-11 June 2015. The sticky inserts of the traps were changed on days 4 and 8 of the experiment, with the treatments re-randomised within the blocks after each change.

Experiment 5 – comparison with other traps. Numbers of midges caught in delta traps baited with lures containing (*R*)-2-nonyl butyrate (0.5 mg) were compared with existing trapping methods, i.e. unbaited sticky traps and water traps. Standard yellow insect sticky traps (25 cm x 10 cm) were mounted on fibreglass canes at crop height. Water traps (Nickerson Brothers Ltd., Lincoln, UK) comprised a yellow bowl (25 cm diameter x 10 cm depth), partly filled with water to which several drops of Fairy dishwashing liquid were added, and mounted on a cane at crop height. All three traps were compared in two 3x3 Latin squares. All traps were checked at weekly intervals between 11-29 May 2015.

231 **Statistical analysis**

232 Numbers of *H. marginata* caught per day for each trap were log(x+1) transformed to improve
233 the homoscedasticity of the data and were analysed using a two-way analysis of variance
234 (ANOVA) with treatment and block as factors. The Least Significant Difference (LSD) test
235 was used to test for significant differences between means at the 5% level. All analyses
236 were done in R.3.2.2 (R Core Team, 2015). Results in Experiment 5 were not analysed
237 statistically due to the extreme heteroscedasticity of the data.

238 ## Results

239 **Pheromone identification**

240 Analyses of collections of volatiles from female *H. marginata* on the non-polar GC column
241 with a male antenna EAG preparation showed one strong EAG response and a weaker
242 response to a compound eluting earlier (Figure 1). Analyses on the polar column showed a
243 strong EAG response but the minor response was not so clear (data not shown). Retention
244 data for the EAG responses and synthetic compounds are shown in Table 1.

245 Analyses of collections of volatiles from female and male *H. marginata* by GC-MS on
246 both non-polar and polar GC columns (Figure 2) showed a female-specific compound that
247 was identified as 2-nonyl butyrate by comparison of retention times (Table 1) and mass
248 spectrum with those of the authentic synthetic compound, and the identification was
249 confirmed by co-chromatography on both GC columns. Up to 50 ng/female of 2-nonyl
250 butyrate was collected during 48 h. This compound had retention data consistent with that
251 of the major response in the GC-EAG analyses (Table 1).

252 2-Nonanol was detected in GC-MS analyses at approximately 2% of the 2-nonyl
253 butyrate. Single ion scanning of the GC-MS analyses of volatiles from female *H. marginata*
254 at *m/z* 71 and *m/z* 89, characteristic of butyrate esters, showed the presence of 2-heptyl
255 butyrate at approximately 1% of the 2-nonyl butyrate. 2-Undecyl butyrate, an analogue
256 reported to be present by Censier et al. (2014), could not be detected (< 0.1% of major

component). Similarly 2,7-dibutyroxynonane, the female sex pheromone of the closely related orange wheat blossom midge, *S. mosellana* (Gries et al., 2000), could not be detected by comparison with the authentic synthetic compound. Other potential minor pheromone components related to 2-nonyl butyrate such as 2-nonanone and 2-nonyl acetate could not be detected (Table 1).

In GC-EAG analyses of the synthetic compounds (10 ng injected), strong EAG responses were observed to 2-nonyl butyrate and 2-heptyl butyrate but there was no detectable response to 2-nonanol (data not shown). The retention indices of 2-heptyl butyrate were consistent with those of the component responsible for the minor EAG responses in analyses of volatiles from female midges on both non-polar and GC columns in the GC-EAG system used (Table 1).

Analysis of the collections of volatiles from female *H. marginata* on the enantioselective cyclodextrin GC column showed a peak at the retention time of (*R*)-2-nonyl butyrate (15.69 min), but no peak (< 5%) at the retention time of the (*S*)-enantiomer (15.30 min).

Pheromone dispensers

Polyethylene vials were found to release 2-nonyl butyrate more uniformly than the rubber septa under laboratory conditions (Figure 3). The rubber septa released over 90% of the pheromone within the first week at 27°C and 2.2 m/sec windspeed. In contrast, 30% of the compound remained after 28 days in the polyethylene vials.

Polyethylene vials containing an initial loading of 1 mg racemic 2-nonyl butyrate and returned from field tests after 2 weeks contained 0.72 ± 0.02 mg (mean ± SEM; N = 3). Polyethylene vials and rubber septa returned from the field after 6 weeks contained 0.41 ± 0.02 mg and 0.31 ± 0.02 mg, respectively.

Field trapping experiments

Experiment 1 – pheromone dispensers. Traps baited with 1 mg racemic 2-nonyl butyrate dispensed from either rubber septa or polyethylene vials caught more male *H. marginata*

than the unbaited traps at site 2 in winter wheat ($F(2,9)=21.33$, $P<0.001$) during the first week of trapping. However, there was no difference in catches with the two dispenser types (Figure 4A). Catches during the next two weeks were too low for analysis but showed the same trend with mean catches per trap over the period of 4.3 ± 1.9 with vials, 5.3 ± 1.4 with septa and no catches in unbaited traps.

Experiment 2 - pheromone chirality. Traps baited with (*R*)-2-nonyl butyrate caught significantly more male *H. marginata* compared to the other treatments ($F(3,9)=22.56$, $P<0.001$). During the 14-d trapping period no adults were caught on the unbaited traps or the traps baited with (*S*)-2-nonyl butyrate, and the catch with racemic 2-nonyl butyrate was less than 5% of that with (*R*)-2-nonyl butyrate (Figure 4B).

Experiment 3 – effect of minor components. A total of 26,658 male *H. marginata* was caught during the 11-d trapping period. Traps baited with racemic 2-nonyl butyrate caught significantly more than unbaited traps but less than 10% of the number caught in traps baited with (*R*)-2-nonyl butyrate (Figure 4C; $F(5,45)=253.66$, $P<0.001$). Addition of the minor components, (*R*)-2-nonanol and/or (*R*)-2-heptyl butyrate did not increase or decrease trap catches compared with catches with the major component, (*R*)-2-nonyl butyrate, alone. There was no interaction between treatment and block but the effect of block was significant ($F(9,45)=6.799$, $P<0.01$).

Experiment 4 – pheromone loading. A total of 13,775 male *H. marginata* was caught during the 9-d trapping period. Significant differences in numbers caught were observed between all treatments ($F(4,36)= 187.42$, , $P<0.001$) and trap catches were dose-dependent with more male *H. marginata* caught when higher pheromone loadings were used (Figure 5A). Log mean catch plotted against log pheromone loading indicated a linear association (Figure 5B).

312 *Experiment 5 – comparison with other traps.* Substantially greater numbers of male *H.*
313 *marginata* were caught in the pheromone trap compared with both the unbaited sticky and
314 water traps. During the trapping period of 18 d with six replicates, over 6,500 *H. marginata*
315 were caught using the pheromone traps compared with 26 and 27 in the sticky and water
316 traps, respectively.

317 ## Discussion

318 Although Censier et al. (2014, 2016a) identified the major component of the sex pheromone
319 produced by female *H. marginata* as (*R*)-2-nonyl butyrate, they only tested the racemic
320 compound in field trapping tests. The racemic compound was reported to be attractive to
321 male *H. marginata*, but the effects of potential minor pheromone components detected were
322 not investigated. Initial studies (Censier et al., 2014) used 20 mg of the racemic compound
323 in polyethylene sachet dispensers with a short field life of several days, and subsequently a
324 rubber septum loaded with 5 mg of the racemic compound was recommended for monitoring
325 (Censier et al., 2016a).

326 Here we confirmed that virgin female *H. marginata* produce (*R*)-2-nonyl butyrate with
327 trace amounts (approx.. 2% relative to the major component) of 2-heptyl butyrate and 2-
328 nonanol. The former two compounds elicited EAG responses from male *H. marginata*, but
329 the latter did not. Censier et al. (2014) reported relatively large amounts of 2-nonanol were
330 detected in volatiles from crushed pheromone glands, presumably due to enzymatic
331 hydrolysis of the butyrate ester (c.f. Ho & Millar, 2002). In field tests, traps baited with (*R*)-2-
332 nonyl butyrate caught more than ten times the numbers of male *H. marginata* caught in
333 those baited with an equivalent amount of the racemic compound. The (*S*)-enantiomer was
334 unattractive, but clearly has an antagonistic effect on the attractiveness of the (*R*)-
335 enantiomer.

336 Absolute configuration is often important in the bioactivity of pheromones (Mori,
337 2007). The sex pheromone components of Cecidomyiid midges identified so far all have

338 one or two chiral centres and the females have been shown or are deduced to produce one
339 stereoisomer (Hall et al., 2012). In components with two chiral centres the correct chirality is
340 invariably critical for attraction. However, in the majority of those with one chiral centre the
341 females produce one enantiomer that is attractive to males but the other enantiomer is
342 neither attractive nor interferes with attraction of the active enantiomer (Hall et al., 2012). *H.*
343 *marginata* is an exception to this trend with the (*S*)-enantiomer not only being unattractive
344 but also reducing the attractiveness of the naturally-produced (*R*)-enantiomer.

345 (*R*)-2-Nonyl butyrate is the simplest midge pheromone reported to date, having an
346 unbranched carbon chain with an odd number of carbon atoms and the characteristic
347 oxygenated functionality at the 2-position (Hall et al., 2012). 2-Nonanol and its esters are
348 relatively easily resolved into the two enantiomers with high enantiomeric excess by kinetic
349 hydrolytic resolution with a lipase enzyme (Hall et al., 2012). The extra cost involved in
350 using (*R*)-2-nonyl butyrate in lures rather than the racemate would probably be outweighed
351 by the more than 10-fold increase in catches, given that the cost of the active ingredient
352 would be a small part of the overall cost of a commercially-produced lure.

353 The absence of any effect of the potential minor pheromone components on
354 attractiveness of the major component was somewhat surprising, although the sex
355 pheromones of many of the cecidomyiid midge species identified to date consist of a single
356 component (Hall et al., 2012). In the chrysanthemum midge, *Rhopalomyia longicauda,* the
357 sex pheromone is (2*S*,8*Z*)-2-butyroxy-8-heptadecene, and addition of even 2% of the
358 corresponding alcohol reduces attractiveness significantly (Liu et al., 2009).

359 Polyethylene vials and rubber septa were compared as commercially-available,
360 practical pheromone dispensers. These were equally effective in field trapping tests and
361 were still attractive after 5 weeks in the field in the UK. However, laboratory tests indicated
362 release from the vials was more uniform than from the septa and the former were likely to
363 last longer in the field. The flight period of *H. marginata* can extend to up to 10 weeks
364 (Censier *et al.* 2015), and the emergence pattern appears to show small peaks of activity
365 throughout the season, coinciding with different growth stages of the crop. The longevity of

366 the lure must therefore allow for the monitoring period to extend over this period. After six
367 weeks in the field polyethylene vials still contained over 40% of the pheromone originally
368 loaded, but the longevity of the lures under field conditions remains to be tested in order to
369 determine the need for lure renewal over entire the *H. marginata* flight period.

370 Increasing the loading of (*R*)-2-nonyl butyrate in polyethylene vial dispensers
371 increased catches of male *H. marginata* with a positive linear association between log mean
372 catch and log pheromone dose over the range tested from 0.05 mg to 2.5 mg. This is useful
373 in estimating the amount of pheromone required for monitoring purposes. Cross and Hall
374 (2009) determined that a mean catch of 25 midges per trap per day was suitable for
375 monitoring apple leaf midge, *Dasineura mali*. The catch rate will inevitably vary greatly
376 depending on the background population and time of trapping, but during the period of this
377 experiment a loading of 0.5 mg of (*R*)-2-nonyl butyrate gave a mean catch rate of 27.3
378 midge per trap per day. These data were obtained mid-season and therefore did not
379 represent the peak catch rate which appears to occur soon after the start of emergence
380 (Censier et al., 2016). The higher loading of 2.5 mg would therefore be unsuitable for
381 monitoring in many field situations as traps would quickly become saturated. Meanwhile the
382 lower loading of 0.05 mg, while still effective during this experiment, may not be sensitive
383 enough to detect early emergence when there are fewer males around.

384 The higher loadings of pheromone may be appropriate if the lures are to be used for
385 control of the pest by mass trapping or lure-and-kill approaches when the maximum catch is
386 required. Use of (*R*)-2-nonyl butyrate rather than the racemic compound might also be
387 advantageous in these situations. For example, a lure containing 2.5 mg of the (*R*)-
388 enantiomer would be equivalent in attractiveness to one containing 50 mg of the racemic
389 compound, and this latter loading would be above the capacity of the dispensers used here.

390 The pheromone has a limitation in that it only monitors male activity and females are
391 rarely caught. This is the same with other successful pheromone trapping systems currently
392 in use for monitoring cecidomyiid pests such as *S. mosellana* (e.g. Bruce et al., 2007).
393 However, unlike *S. mosellana* where females disperse after mating, there is little evidence

for female dispersal in *H. marginata*. Skuhravý et al. (1983) observed that females made several short flights until a suitable host plant was found. The same study also recorded a sex ratio of females to males of 59:41 and 54:46 based on emergence trap and Möricke trap catches respectively. Female numbers are therefore likely to be slightly higher than, or comparable to the number of males being caught. In practice, the enhanced performance of these pheromone traps in comparison to existing methods for trapping cecidomyiids far outweighs this limitation.

The present study indicates that a polyethylene vial loaded with 0.5 mg of (*R*)-2-nonyl butyrate is a suitable lure for trapping adult *H. marginata* in the field, and would be equivalent in attractiveness to a lure containing 10 mg of the racemic compound. This system will greatly improve detection in areas of low *H. marginata* populations, and will provide a greater degree of accuracy when monitoring for the start of adult activity. Further work is required to confirm the longevity of the lure under field conditions and to establish the relationship between trap catches and crop damage in order to provide a threshold above which treatments should be applied.

Acknowledgements

The authors are grateful to AHDB Cereals & Oilseeds for funding this work (Project Number 214-0002) and to the farmers for the use of their land for field trials.

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505

506 **Table 1** Retention indices relative to retention times of *n*-alkanes of EAG responses in GC-
507 EAG analyses of volatiles from virgin female *Haplodiplosis marginata* with male *H. marginata*
508 EAG preparation, and of synthetic compounds

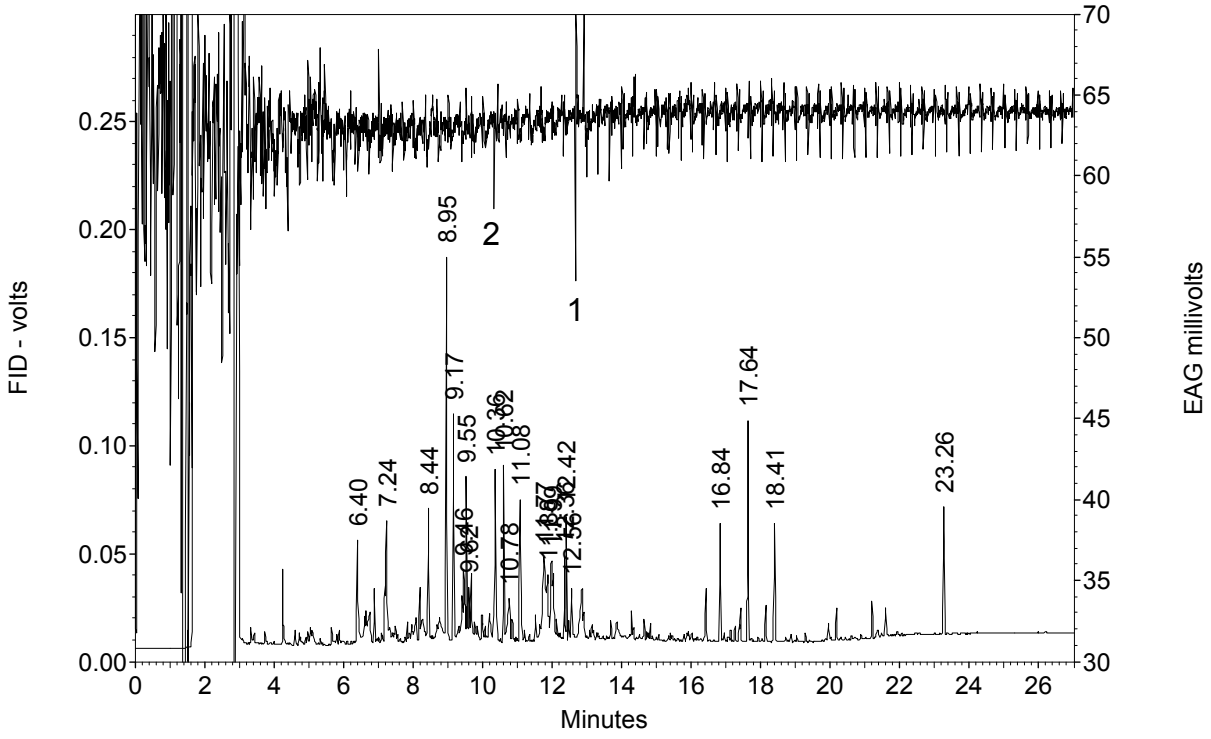
	Non-polar		Polar	
	GC-EAG (SPB1)	GC-MS (VF5)	GC-EAG (DBWax)	GC-MS (DBWax)
EAG major	1389		1601	
EAG minor	1235		1415	
2-nonyl butyrate	1389	1403	1601	1591
2-nonanol	1082	1104	1528	1513
2-nonyl acetate	1218	1234	1460	1456
2-heptyl butyrate	1201	1215	1400	1392
2,7-dibutyroxy-nonane	1846	1861	2282	2245
2-nonanone	1075	1092	1376	1378

509
510

511 **Figure 1** Coupled GC-EAG analysis of collection of volatiles from female *Haplodiplosis*
512 *marginata* on non-polar column (lower is expansion of upper; major response (1) to 2-nonyl
513 butyrate at 12.42 min; minor response (2) at 10.2 min; 2-heptyl butyrate at 10.00 min, 2-
514 nonanol at 8.25 min).
515
516 **Figure 2** Coupled GC-MS analyses on polar GC column of volatiles from female
517 *Haplodiplosis marginata* (upper) and volatiles from male *H. marginata* (lower) ((1) 2-
518 nonylbutyrate; (2) 2-nonanol; (3) 2-heptyl butyrate)
519
520 **Figure 3** Release of 2-nonyl butyrate (1 mg) from rubber septa and polyethylene vials in
521 laboratory wind tunnel at 27 °C and 2.2 m/sec wind speed as measured by GC analyses of
522 the amount remaining at intervals.
523
524 **Figure 4 (A)** Catches of male *Haplodiplosis marginata* in traps baited with racemic 2-nonyl
525 butyrate (1 mg) dispensed from polyethylene vials or rubber septa (Experiment 1, 15-21 May
526 2014; means with different letters are significantly different $P < 0.001$). **(B)** Catches of male
527 *H. marginata* in traps baited with racemic 2-nonyl butyrate (1 mg), (*R*)-2-nonyl butyrate (0.5
528 mg), (*S*)-2-nonyl butyrate (0.5 mg) and unbaited (Experiment 2, 5-19 June 2014; means with
529 different letters are significantly different $P < 0.001$. **(C)** Catches of male *H. marginata* in
530 Experiment 3 (18-29 May 2015; treatment A 0.5mg (*R*)-2-nonyl butyrate, B 0.5mg (*R*)-2-
531 nonyl butyrate + 2% (*R*)-2-nonanol, C 0.5mg (*R*)-2 nonyl butyrate + 2% (*R*)-2 heptyl-
532 butyrate, D 0.5mg (*R*)-2-nonyl butyrate + 2% (*R*)-2-nonanol + 2% (*R*)-2 heptyl-butyrate, E
533 1mg racemic 2-nonyl butyrate, F unbaited control; bars show back-transformed means ±
534 SEM; means with different letters are significantly different at $P<0.05$ by LSD test).
535
536 **Figure 5 (A)** Catches of male *Haplodiplosis marginata* in Experiment 4 with different lure
537 loadings of (*R*)-2-nonyl butyrate and unbaited control (2–11 June 2015; bars show back-
538 transformed means ± SEM; means with different letters are significantly different at $P<0.05$

539 by LSD test). (**B)** Plot of log mean catch per trap per day of *H. marginata* against log
540 pheromone loading in Experiment 4.
541

542



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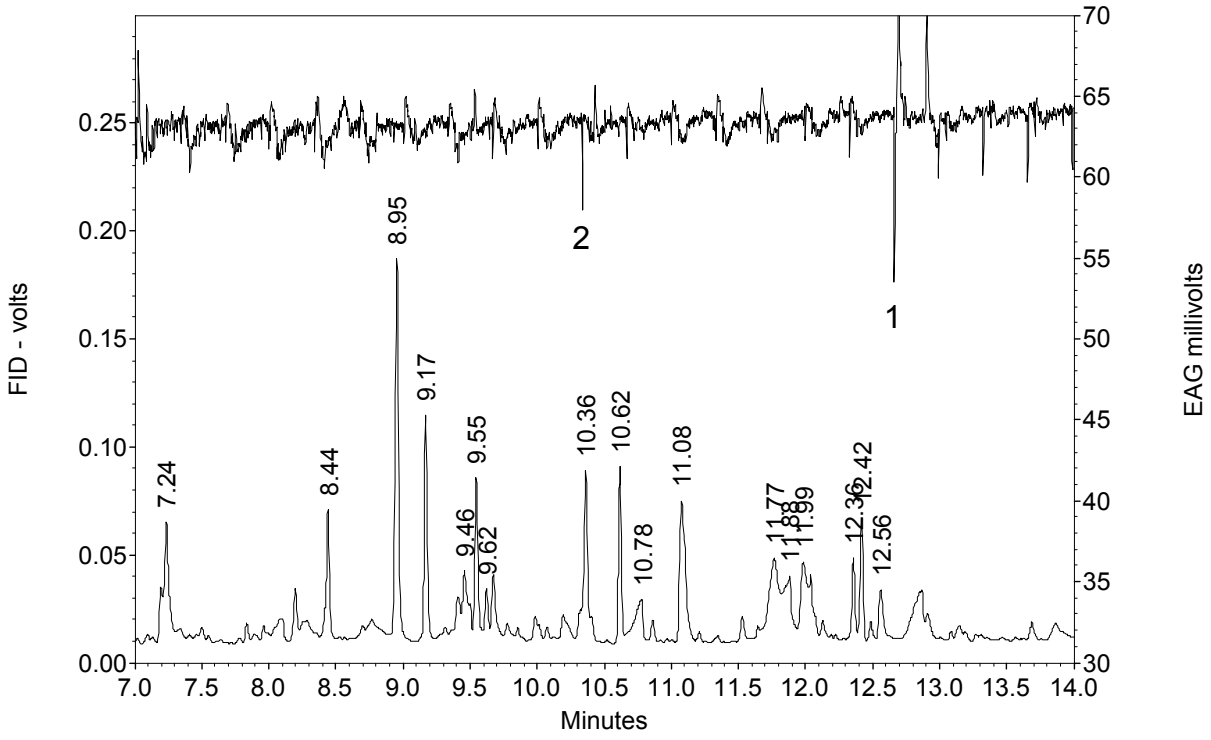

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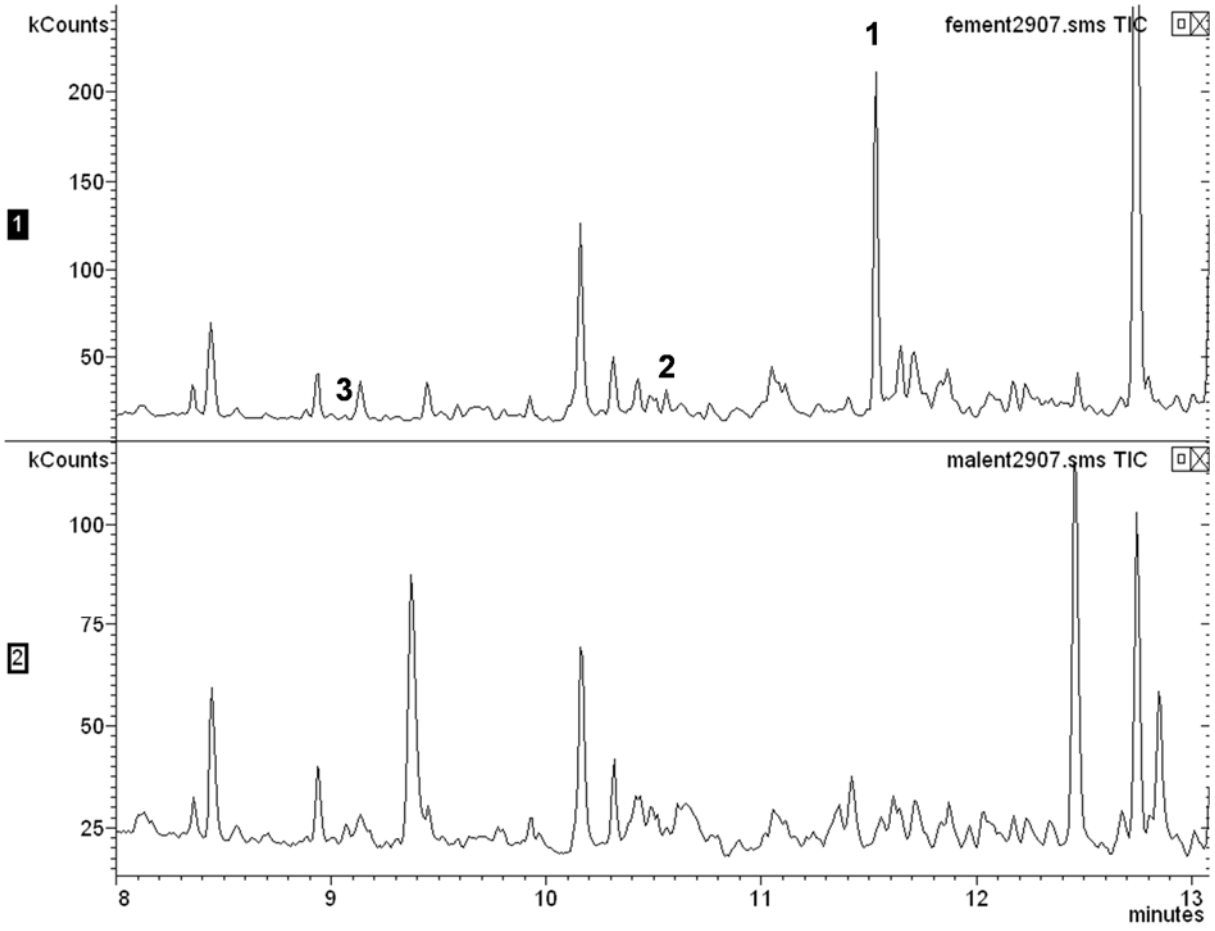
kCounts
fement2907.sms TIC
1
200
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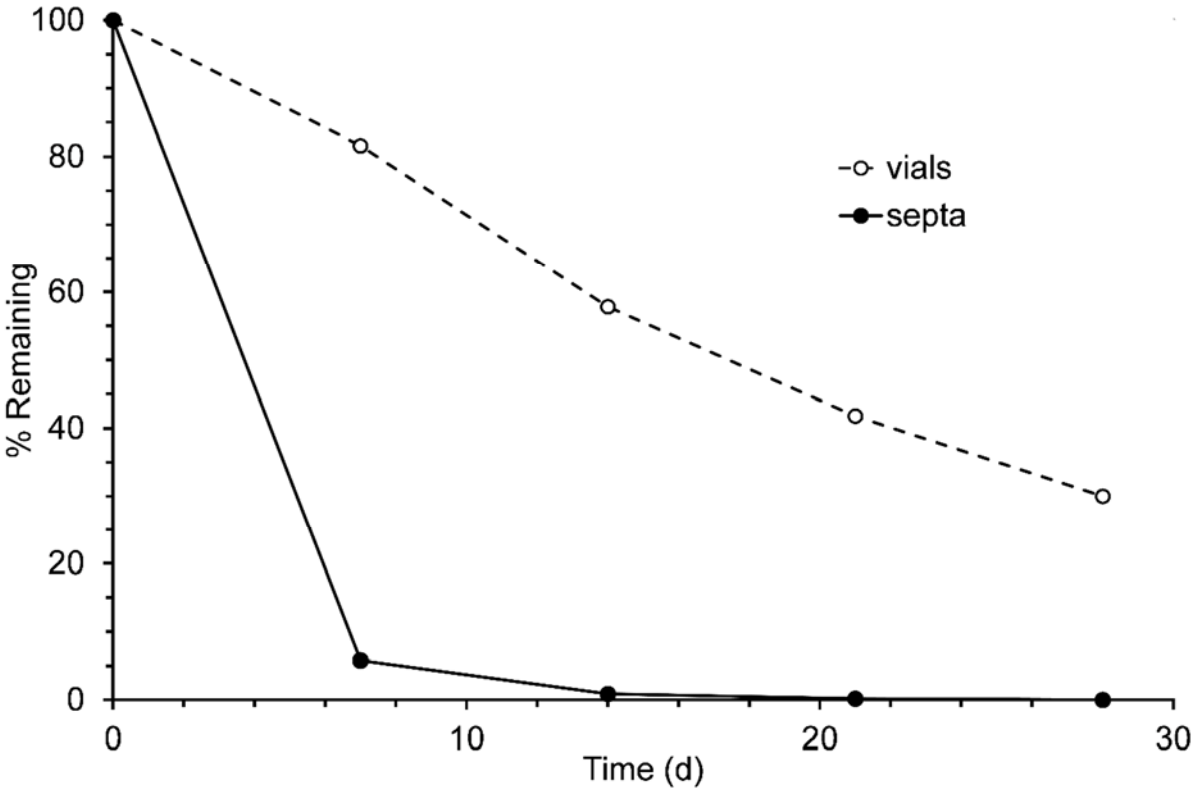

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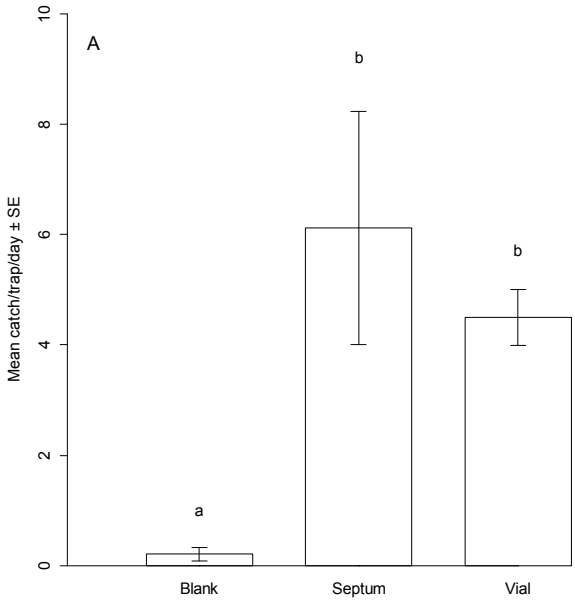
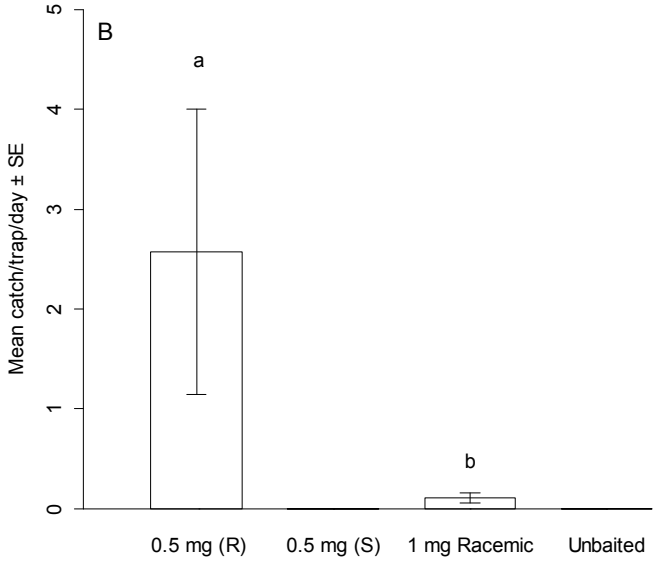
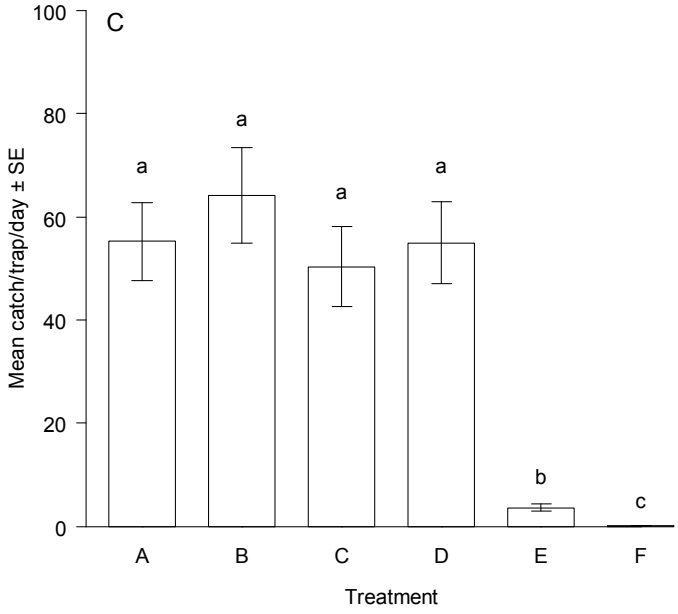

A
Mean catch/trap/day ± SE
0
2
4
6
8
10
a
b
b
Blank
Septum
Vial

552

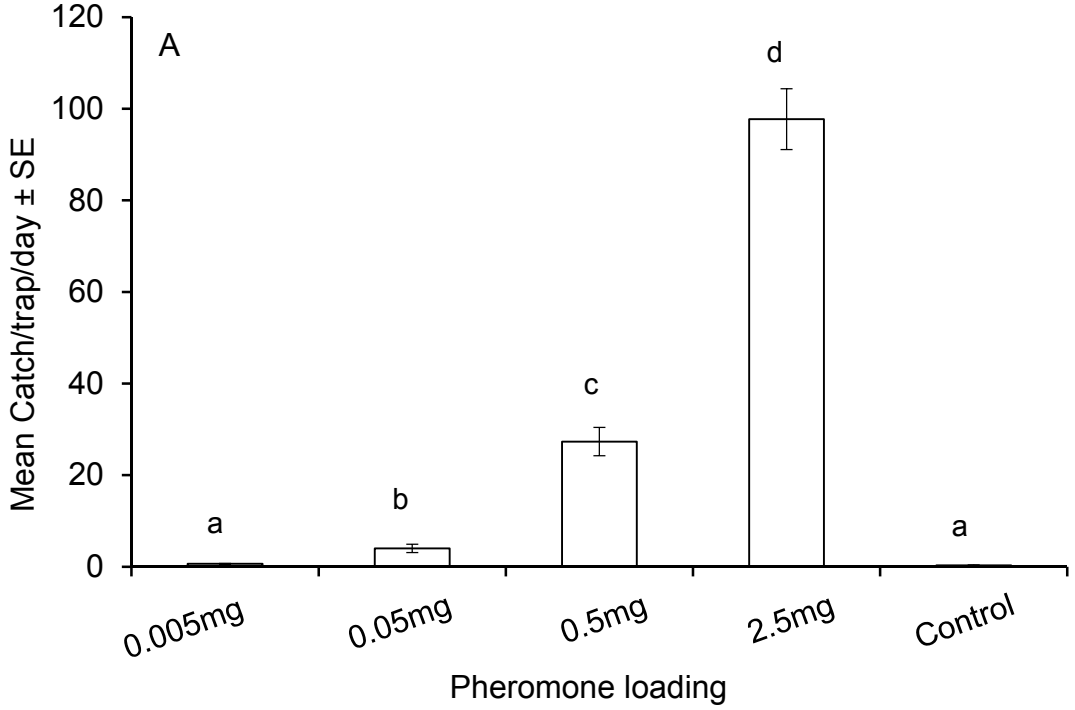

B
Mean catch/trap/day ± SE
0
1
2
3
4
5
a
b
0.5 mg (R)
0.5 mg (S)
1 mg Racemic
Unbaited

553


C
Mean catch/trap/day ± SE
0
20
40
60
80
100
a
a
a
a
b
c
A
B
C
D
E
F
Treatment

554

555



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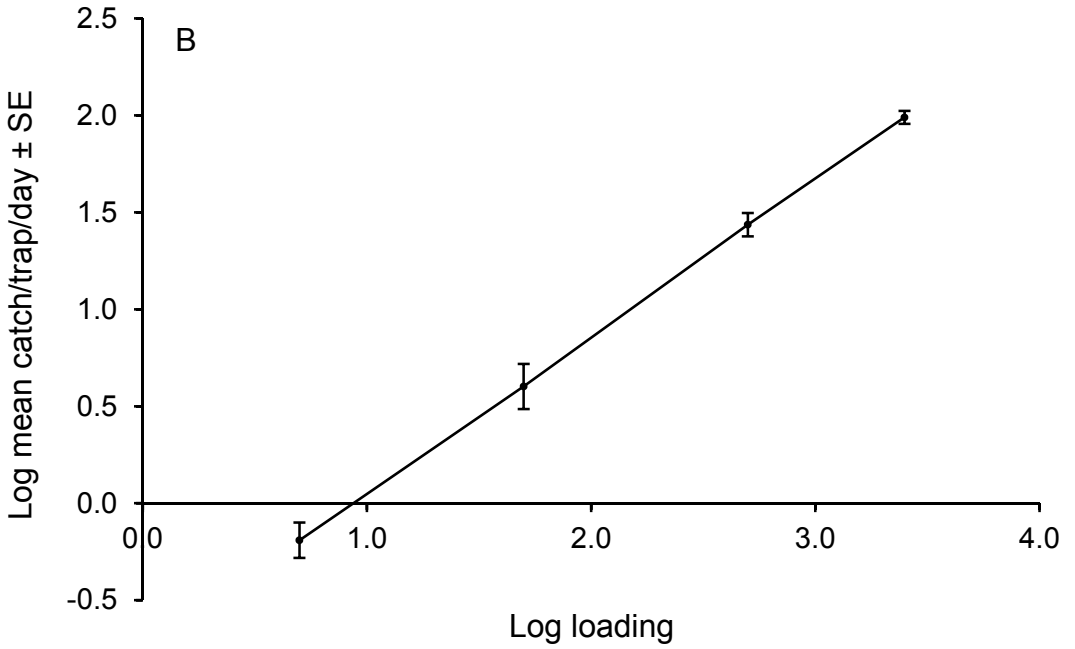

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