Identification of the Male-Produced Aggregation Pheromone of the Four-Spotted Coconut Weevil, *Diocalandra frumenti*

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ABSTRACT

The four-spotted coconut weevil, *Diocalandra frumenti* Fabricius (Coleoptera: Curculionidae), is a small weevil found attacking economically important palm species such as coconut, date, oil and Canary palms. Given the scarcity of detection and management tools for this pest, the availability of a pheromone to be included in trapping protocols would be a crucial advantage. Previous laboratory experiments showed evidence for aggregation behavior; thus, our main goal was to identify the aggregation pheromone in this species. The volatile profile of *D. frumenti* individuals was studied by aeration and collection of effluvia in Porapak-Q and also by solid phase microextraction (SPME) techniques. Moreover, solvent extraction of previously frozen crushed individuals was also performed. All resulting extracts and SPME fibers were analyzed by gas chromatography coupled to mass spectrometry (GC-MS). The comparison of male and female samples provided the candidate compound, 5-ethyl-2,4-dimethyl-6,8-dioxabicyclo[3.2.1]octane (multistriatin), whose biological activity was evaluated in olfactometer and field assays.

KEYWORDS

Diocalandra frumenti; aggregation pheromone; semiochemical; chemical ecology; attractant; weevil; Coleoptera; Curculionidae; Dryophthoridae

INTRODUCTION

The four-spotted coconut weevil, *Diocalandra frumenti* Fabricius (Coleoptera: Curculionidae), is a small black weevil (6-8 mm in length) with four large reddish spots on the elytra.[1] Native to Asian coastal areas of the Indian Ocean (Bangladesh, India, Indonesia, Thailand…), it is also reported in Madagascar, Tanzania, Australia, Pacific Islands, Japan, Ecuador and more recently in Canary Islands (Spain).[2] It is found attacking at least 17 genera of Arecaceae, most of these being economically important palm species cultivated for food or landscape purposes, such as *Cocos nucifera* L., *Phoenix dactylifera* L., *Phoenix canariensis* Hort. ex Chabaud or *Elaeis guinensis* Jacq.[1-3] Larvae are found mainly attacking leaves, where they bore into the tissues opening galleries and causing gum exudation. The leaf bases are bored from the trunk out to the leaflets, which may cause yellowing and collapse of fronds, beginning from the exterior fronds and moving to the interior ones.

Given that this species is cryptic and is therefore difficult to detect, symptoms for infestation are emergence holes, frond collapse, premature fruit fall and gum exudate. There is not any specific chemical registered to control *D. frumenti* but chemical control is mainly based on the use of chlorpyrifos and imidacloprid.[4] Cultural practices are also important to manage this pest, such as removing and destroying old and dead fronds to reduce breeding sites and avoiding pruning in the warmest season. Since its introduction in Gran Canaria in 1998,[2] *D. frumenti* has rapidly expanded to other islands of the Canarian Archipelago causing a dramatic increase in palm infestation. Given the scarcity of detection and management tools for this pest, the availability of a pheromone to be included in trapping protocols would be a crucial advantage to restrain its invasion.

Trapping is currently based on non-specific attractants such as the use of bucket type traps baited with sugarcane pieces and water. Unfortunately, plant material has low attractant power by itself but, usually, synergize the effect of aggregation pheromones.[5] As far as we know no studies on detection and/or identification of an aggregation pheromone in *D. frumenti* are reported. Aggregation pheromones for 30 species of weevils in the Curculionoidea superfamily have been reported.[6,7] Previous experiments revealed that *D. frumenti* male and female were attracted to live males in an olfactometer. Thus, our goal was to determine whether this species produced an aggregation pheromone. Different techniques of volatile collection and extraction were employed to study the volatile profile of *D. frumenti* male and female. Their comparison provided a candidate compound, whose biological activity was evaluated in olfactometer and field assays.

MATERIALS AND METHODS

Weevils. Insects were collected from damaged *P. canariensis* palms located in Gran Canaria (Canary Islands, Spain), trapped in modified funnel traps baited with sugarcane and water. Weevils were taken to laboratory, separated by sex and placed inside plastic containers. Insects were maintained until use in a rearing chamber, in darkness at 22±2 ºC, 80% relative humidity and provided with water and sugarcane.

Collection of volatiles. Three groups of either 50-60 males or females were placed in three 2 L-glass round bottom flasks, with 10 g sugarcane/each, connected in parallel to a single glass cartridge to trap all the released volatiles in 3 g Porapak-Q (Supelco Inc., Torrance, CA). Samples were collected continuously over 7 d in darkness by using

a 0.2 L/min charcoal-filtered airstream. Trapped volatiles were then extracted with 20 mL pentane (Chromasolv, Sigma-Aldrich, Madrid, Spain) and the extracts were concentrated to 500 µL under helium stream prior to chromatographic analysis.

In addition, groups of either 40 males or females were placed in closed 5 mL-glass vials with a piece of moistened filter paper for 3 d. A sample of the vial headspace was taken with a SPME holder equipped with a polydimethylsiloxane/divinylbenzene fiber (PDMS/DVB; 100 µm film thickness) (Supelco Inc., Torrance, CA). SPME fibers were conditioned before volatile sampling in a gas-chromatograph (GC) injection port at 250 °C for 10 min with a helium flow rate of 20 mL/min. For the sampling, SPME needle was inserted through a septum and the fiber was exposed to each sample headspace for 12 h at 25 ± 1 °C. After this period, fibers were removed and inserted into the GC injection port to desorb volatiles for the chromatographic analysis.

Solvent extraction of weevils and crude fractionation. A study of weevil volatile profiles was also done in parallel by solvent extraction of previously frozen individuals. Groups of either 1000-2000 males or females were frozen at -50 ºC, crushed and extracted by soaking in 100 mL pentane with magnetic agitation for 24 h. Each crude extract obtained by careful removal of the solvent was fractionated by gravity column (500 mm x 35 mm i.d.) using silica gel as stationary phase (40-60 µm). The column was successively eluted with 75 mL each of 0, 2, 20 and 100% diethyl ether in pentane. Thirty fractions of approx. 10 mL were collected and the volatile profile of each fraction was studied by SPME with PDMS/DVB fibers after careful removal of the solvent using a gentle nitrogen stream. In this way, we avoided injecting in the GC the heaviest compounds of each fraction, which were assumed not to include volatile or semi-volatile substances susceptible of being pheromonal compounds. Each fraction was

placed in 20-mL headspace glass vials with PTFE/silicone septum crimp caps (Teknokroma SL, Barcelona, Spain). A sample of the vial headspace was taken for 6 h at 45 ± 1 °C, to assist volatilization of compounds. After this period, fibers were removed and inserted into the GC injection port to desorb volatiles for the chromatographic analysis.

Chromatographic and Spectroscopic Analysis. All resulting pentane extracts and SPME fibers were analyzed by gas chromatography coupled to mass spectrometry (GC-MS) using a Clarus 600 GC-MS (PerkinElmer Inc, Waltham, MA). SPME fibers were desorbed for 1 min into the GC injection port set in splitless mode at 250 ºC. The column used was a 30 m x 0.25 mm i.d., 0.25 μm, ZB-5MS fused silica capillary column (Phenomenex Inc., Torrance, CA). The oven was held at 40 °C for 2 min and then programmed at 5 °C/min to 180 °C and, when reached, raised to 280 °C at 10 °C/min and maintained at 280 °C for 1 min. Helium was used as the carrier gas with a flow of 1 mL/min. The detection was performed in the EI mode (70 eV) with the ionization source set at 180 ºC. The spectrum acquisition was performed in scanning mode (mass range *m/z* 35-500) and chromatograms and spectra were recorded by means of GC-MS Turbomass software v. 5.4 (PerkinElmer). Compounds were identified by comparing their retention indices and mass spectra with those of pure standards and high probability matches (>80%) according to the NIST MS Search routine (NIST Mass Spectral Search Program for the NIST\EPA\NIH mass Spectral Library, version 2.0, build 4/2005).

^{1}H Nuclear magnetic resonance (NMR) spectra of synthetized compounds were recorded on a AV-300 spectrometer (Bruker, Billerica, MA) at a frequency of 300 MHz

and ^{13}C spectra at a frequency of 75 MHz. Deuterochloroform ($CDCl_3$) was used as solvent with tetramethylsilane (TMS) as internal standard.

Synthesis of 5-ethyl-2,4-dimethyl-6,8-dioxabicyclo[3.2.1]octane (1). A non-stereospecific synthesis of the four pairs of enantiomers of multistratin **1** (Figure 1) was carried out following the synthetic route described by Pearce et al.[8] A sample of the final product was purified by gravity column (300 mm x 20 mm i.d.) using silica gel (40-60 µm) as stationary phase and a mixture of hexane:diethyl ether (8:2, v/v) as eluent. Four peaks were detected by GC-MS chromatography with retention times (t_R) of 18.59, 19.00, 19.36, 19.63 min (Figure S2), and relative areas of 68:25:5:2, which were identified as **1** stereoisomers by high probability matches to 5-ethyl-2,4-dimethyl-6,8-dioxabicyclo[3.2.1]octane (Figure 1). The mixture of stereoisomers was initially assigned as δ:α:γ:β, according to the elution order and relative percentages described in the literature.[8] Proton (^1H) (Figure S3) and carbon (^{13}C) NMR data (Figure S4) of this sample were in agreement with those previously reported in the literature.[9,10] The most intense ^{13}C NMR signals of this mixture were unequivocally assigned to the (±)-δ-**1** stereoisomers (Figure S5 and S6). Careful chromatography of the sample using pentane:diethyl ether mixture (98:2) as eluent afforded an enriched fraction of the stereoisomers with t_R = 19.00 min (δ:α:γ relative areas 19:70:11 by GC/MS) (Figure S7). The most intense ^{13}C NMR signals of this mixture were unequivocally assigned to the (±)-α-**1** stereoisomers (Figure S10 and S11), confirming the previously assigned elution order of the stereoisomers. Representative mass spectra of the stereoisomers *m/z* (%): 170 (M+, 5), 128 (6), 96 (7), 81(5), 71 (7), 57 (100), 55(12), 41 (6). (±)-δ-**1** (t_R = 18.59 min): ^{13}C NMR (75 MHz, CDCl3) δ 111.35, 78.80, 69.88, 33.13, 32.94, 32.42, 27.26, 17.86, 16.36, 7.04; (±)-α-**1** (t_R = 19.00 min): ^{13}C NMR (75 MHz, $CDCl_3$) δ

110.37, 78.86, 65.20, 37.19, 34.83, 32.94, 27.26, 16.80, 16.77, 7.18; (±)-γ-**1** (t_R = 19.36 min) ^{13}C NMR (75 MHz, $CDCl_3$) δ 110.37, 68.86, 64.25, 35.72, 33.43, 28.39, 27.29, 16.76, 16.28, 6.97.

Chemicals. All reagents and solvents (reagent grade) were purchased from Sigma-Aldrich (Madrid, Spain) and employed without additional purification unless stated. All organic solvents used in experiments were dried with appropriate drying agents and distilled before use.

Laboratory behavioral tests. Given that *D. frumenti* is considered a quarantine pest all biological tests were carried out in Canary Islands. Unfortunately, electroantennographic (EAG) tests could not be performed due to the lack of the required equipment in that location and the quarantine restrictions to carry the insects to peninsular Spain.

Male and female *D. frumenti* responses to the synthesized mixture of multistriatin diastereomers were tested in a 2-choice Y-tube glass olfactometer (ARS Inc., Gainesville, FL), using charcoal-filtered air at 0.8 L/min. The olfactometer consisted of a glass tube (16-cm long × 1.8-cm diam.) with two 18-cm arms. Odor sources were placed at the end of each arm and consisted of a 1-cm^2 piece of filter paper over a 20×20-mm glass microscope slide to avoid direct contact of the odor with the olfactometer. Filter paper pieces were loaded with 20 μL of the corresponding dilutions to test different doses of the synthetic mixture of multistriatin: 0.001, 0.1, 10 and 100 μg. Control stimuli consisted of filter paper pieces loaded with 20 μL pentane.

All assays were conducted in darkness at 25 ± 2 °C and 75% relative humidity, using a red light to monitor weevil responses. It was previously ascertained that there was no difference in responses of males or females in the olfactometer when both arms were blank, indicating a lack of positional effect of the experimental setup. Groups of weevils were transported from the rearing chamber to the bioassay room 24 h before the tests, under darkness and provided only with water. In each test, a single weevil was placed at the entrance of the main olfactometer tube, and response to the corresponding stimulus was observed for 10 min. Weevil behavior was recorded as: pheromone choice or control choice (they visited the respective arm one or several times), no choice (they moved but did not reach any arm or visited both) and non–responding (they were inactive and did not leave the starting point). After the test, the insect was discarded so each test employed a different weevil. In general, the effect of each stimulus was tested with 40 weevils/sex (N = 40). The position of the stimulus was shifted and the olfactometer rotated 180º every 5 individuals of the same sex to avoid effects caused by possible weevil tracks.

The null hypothesis that *D. frumenti* showed no preference for either olfactometer arm (response equal to 50:50 for stimulus:solvent) was analyzed with a Chi-square goodness of fit test with SPSS 16.0.1 statistical package (SPSS Inc., Chicago, IL).

Field trials. Male and female *D. frumenti* responses to the synthetic mixture of multistriatin were also tested in field conditions. The substances were emitted from rubber septa, which were loaded by impregnation with a hexane solution of synthetic multistriatin and allowing solvent to evaporate. Traps employed were green funnel traps (Econex SL, Murcia, Spain), modified by drilling two 2.5-cm diameter opposite holes around the bucket to facilitate weevil entrance, based on previous experiments using

sugarcane as bait. Rubber septa were hung in the center of the trap, whose base was filled with 500-mL of water as retention system. Sugarcane (four 15-cm pieces) was also included in the traps according the description of the trials detailed below.

The trials were carried out in Campo Internacional, Maspalomas (Gran Canaria Island, Spain) (coordinates: 27°45'08.6"N, 15°35'33.9"W). Trial area was a residential zone with groups of ornamental *P. canariensis* and hybrid palms (*P. canariensis* x *P. dactylifera*), but only the first were selected for trials. Traps were set on the crown of the palm trees as suggested by preliminary field tests performed with traps baited with water and sugarcane.

In Trial 1, three blocks of three traps were placed in a row inside each block using a randomized complete block design. Therefore, each block contained a trap baited with: (1) water+sugarcane, (2) water+sugarcane+1-mg synthetic multistriatin impregnated rubber septum and (3) water+sugarcane+5-mg synthetic multistriatin impregnated rubber septum. Traps were installed in palm trees at least 20 m apart, from 30 January to 22 February 2016 (main climate features: Tmax = 20.5 ºC, Tmin = 14.5 ºC, Tmean = 17.3 ºC, HRmean = 61.3%) and were examined weekly for three weeks. All baits, including rubber septa, were replaced every week and traps rotated inside each block such that each bait was tested at each position.

Traps in Trial 2 were arranged using a randomized complete block design, with four blocks of three traps. In this case, each block contained a trap baited with: (1) water+sugarcane, (2) water+1-mg synthetic multistriatin impregnated rubber septum and (3) water+sugarcane+1-mg synthetic multistriatin impregnated rubber septum. Traps were installed in palm trees at least 20 m apart, from 8 March to 29 March 2016 (main climate features: Tmax = 22.0 ºC, Tmin = 15.1 ºC, Tmean = 18.5 ºC, HRmean =

59.7%). Examination of captures, rotation of traps and replacement of baits was made on a weekly basis, as detailed above.

The number of *D. frumenti* males and females captured per trap and day were log-transformed to homogenize variance prior to applying the analysis of variance (ANOVA). A multifactor ANOVA (Fisher least significant difference [LSD] test at $P < 0.05$) was performed to study the effect of three factors (week, block and trap bait) on weevil captures. When significant effect of trap bait was found, Tukey's HSD test was employed for post hoc pairwise comparisons. These analyses were conducted using the Statgraphics Centurion XVI package (StatPoint Technologies, Warrenton, VA).

RESULTS AND DISCUSSION

Chemical Analysis. The chromatographic volatile profile of male and female *D. frumenti* from Gran Canaria showed a male-specific compound at t_R = 19 min (Kovats index = 1135 on the ZB-5 column) (Figure 2), which was detected in both static and dynamic headspace collections. This compound was also found in the volatile profile of fraction 23 of male crude extract. The mass spectrum of this compound had a base peak at *m/z* 57, and fragments at *m/z* 71, 81, 86, 96, 128, 140, 170. When the mass spectrum was compared to the NIST Spectral library, it was matched with high probability to 5-ethyl-2,4-dimethyl-6,8-dioxabicyclo[3.2.1]octane, also known as multistriatin **1** (Figure 1).

The compound was identified as (±)-α-**1** by co-injection of the Porapak-Q extract with the synthetic 5-ethyl-2,4-dimethyl-6,8-dioxabicyclo[3.2.1]octane mixture with a relative area 68:25:5:2 of the δ:α:γ:β stereoisomers. Identification was based on the coincidence of retention time (t_R = 19.00 min) and MS spectrum of the previously

assigned (±)-α-**1** stereoisomers. The isomer (-)-α-**1** was described as component of the aggregation pheromone emitted by the females of the bark beetle *Scolytus multistriatus* (Marsham) (Coleoptera: Curculionidae: Scolytinae).[11] Moreover, the bicyclic ketal core structure of multistriatin is identical to those of two other bark beetle aggregation pheromones, brevicomin **2** (7-ethyl-5-methyl-6,8-dioxabicyclo[3.2.1]octane) and frontalin **3** (1,5-dimethyl-6,8-dioxabicyclo[3.2.1]octane), isolated from *Dendroctonus brevicomis* LeConte and *D. frontalis* Zimmerman (Figure 1).[12,13] Interestingly, a closely related ring structure was found in the aggregation pheromone of the banana weevil *Cosmopolites sordidus* Germar (Coleoptera: Dryophthoridae: Rhynchophorinae), sordidin **4** (1-ethyl-3,5,7-trimethyl-2,8-dioxabicyclo[3.2.1]octane) (Figure 1).[14] Generally, palm weevil aggregation pheromones have aliphatic methyl-branched secondary alcohol structures, including those belonging to the genera *Rhynchophorus* and *Dynamis*,[15-19] such as ferrugineol **5** (4-methyl-5-nonanol) (Figure 1), but this was not the case of *D. frumenti* which is also considered a palm weevil.

Laboratory behavioral tests. In general, some insects moved directly towards the odor source, and others made several turns in the main arm of the olfactometer before making a choice. In summary for the total number of weevils (females + males) employed for these behavioral tests, 24.7% of the weevils did not choose any of the olfactometer arms or visited both of them, 13.8% did not reach any and 5.0% were inactive and did not leave the starting point (non-responding weevils) (Figure 3).

Weevils responded differently to synthetic multistriatin depending on the tested dose (Figure 3). Considering only those insects that chose either olfactometer arm, weevils significantly preferred synthetic multistriatin compared to the blank stimulus when it was provided at doses of 10 and 0.1 µg (Table 1). Response of males to a 100 µg dose

was not significant, whereas females did prefer significantly the arm baited with synthetic multistriatin (Table 1). The lowest dose of 0.001 µg did not trigger any significant response of females or males; indeed, the percentage of weevils that choose either arm was the lowest in those tests (only 15-16 out of 40 weevils of each sex reached either arm). This could be due to the low dose of the active stereoisomer that was actually reaching the olfactory system of the weevils, given that we were employing a mixture of four pairs of enantiomers.

Field trials. In Trial 1, the type of bait employed had a significant effect on weevil captures (Table S1), for females, males and total weevils (Figure 4A). The addition of synthetic multistriatin to traps baited with sugarcane improved weevil captures regardless the dose tested (rubber septa loaded with 1 or 5 mg). The combination of the aggregation pheromone with natural kairomone odors, in this case provided by sugarcane pieces, had a strong synergistic effect on *D. frumenti* attraction. The block factor also had a significant effect on captures due to the aggregated distribution of the pest; likewise, week factor was also significant (Table S1) probably due to the natural population dynamics. Interactions between the factors studied were also considered and were not significant in all cases (Table S1). Regarding the sexual dimorphism in the response of weevils, traps baited with sugarcane attracted as many males as females (mean ratio females/males (f/m): sugarcane = 1.0). However, a little predominance of females was observed when bait included synthetic multistriatin (mean f/m: sugarcane + 1-mg multistriatin = 1.1; sugarcane + 5-mg multistriatin = 1.3).

The attractant power of synthetic multistriatin by itself was also evaluated in Trial 2. The type of bait employed had a significant effect on *D. frumenti* captures (Table S1), both for females, males or total weevils (Figure 4B). Sugarcane and synthetic

multistriatin had a lower attractant effect by themselves, capturing significantly fewer weevils than their combination. In fact, the four traps baited only with synthetic multistriatin captured a total of 9 weevils (females + males) during the 3 weeks of trial and those baited only with sugarcane captured a total of 169 weevils. On the other hand, the combination of sugarcane + synthetic multistriatin captured 803 weevils, 4.7 and 89 times more weevils than the single baits respectively, again demonstrating their synergistic effect. This is consistent with reports of the synergistic attraction of aggregation pheromones and odors of plant tissues both for species of Rhynchophorinae and Scolytinae.[11,19-21] Plant odors boost the attractiveness of the aggregation pheromone and act as arresting or retaining agents. When plant kairomones are absent, weevils are attracted by the pheromone to the vicinity of the trap but either fail to enter or escape after entering, with the consequent risk of attacking neighboring palm trees.

As observed in Trial 1, sugarcane attracted as many males as females (mean f/m = 1.0) also in Trial 2, whereas its combination with synthetic multistriatin attracted more females than males (mean f/m = 1.8 respectively). In the olfactometer, female *D. frumenti* responded to the synthetic mixture in a wide range of doses (from 100 to 0.1 µg), whereas response of males was more limited. Thus, we have observed both in laboratory and field experiments a predominant female response to multistriatin, which could be related to the female-biased response to aggregation pheromones displayed by some palm weevils.[22,23] In the case of *R. ferrugineus*, wild populations may be naturally female-biased because the ~2:1 ratio (f:m) has been observed both by catches in pheromone-baited traps and by inspection of infested Canary palms.[22] In our field trials, although *D. frumenti* females and males responded equally to sugarcane, there was a predominance of female captures in response to baits that included synthetic multistriatin, evidencing the sexual bias in pheromone response.

Our data demonstrate that *D. frumenti* males produce an aggregation pheromone as do other palm weevil species. Elucidation of the absolute stereochemical configuration of the aggregation pheromone and synthesis of a pure sample of α-multistriatin is still ongoing. Responses of insects that employ bicyclic ketals as aggregation pheromones are rarely inhibited by the presence of enantiomers and diastereomers of their natural semiochemicals.[24,25] This has important consequences for practical purposes because fortunately, both our laboratory and field experiments demonstrated the attractant power of the affordable isomeric mixture synthesized. The identification of *D. frumenti* aggregation pheromone is a considerable step towards the surveillance, monitoring and control of this pest, which is threatening the endemic palm trees of Canary Islands.

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SUPPORTING INFORMATION

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Table S1. Statistical data of the trials carried out to evaluate the effect of synthetic multistriatin on *Diocalandra frumenti* captures. Significance of the studied factors by ANOVA, LSD test at $P < 0.05$.

Figure S1. Representative GC/MS spectra of multistriatin

Figure S2. GC/MS chromatogram of synthetic mixture of multistriatin stereoisomers

Figure S3. ^{1}H NMR spectrum of synthetic mixture of multistriatin stereoisomers

Figure S4. ^{13}C NMR spectrum of synthetic mixture of multistriatin stereoisomers

Figure S5. ^{13}C NMR with assigned signals for δ, α and γ stereoisomers (part 1)

Figure S6. ^{13}C NMR with assigned signals for δ, α and γ stereoisomers (part 2)

Figure S7. GC/MS chromatogram of the α-enriched synthetic mixture of multistratin stereoisomers

Figure S8. ^{1}H NMR of the α-enriched synthetic mixture of multistratin stereoisomers

Figure S9. ^{13}C NMR of the α-enriched synthetic mixture of multistratin stereoisomers

Figure S10. ^{13}C NMR with assigned signals for δ, α and γ stereoisomers (in the α-enriched multistriatin mixture) (part 1)

Figure S11. ^{13}C NMR with assigned signals for δ, α and γ stereoisomers (in the α-enriched multistriatin mixture) (part 2)

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FIGURE CAPTIONS

Figure 1. Chemical structures of some aggregation pheromones of bark beetles and weevils (multistriatin, **1**, brevicomin, **2**, frontalin, **3**, sordidin, **4**, and ferrugineol, **5**) and stereoisomers of multistriatin, **1**.

Figure 2. Representative GC/MS chromatograms of *Diocalandra frumenti* volatile profiles: (A) females and (B) males, compared with (C) synthetic mixture of multistriatin **1** stereoisomers. Male-specific compound at 19.00 min matched retention time of (±)-α-**1**.

Figure 3. Behavioral response of (A) female and (B) male *Diocalandra frumenti* in a Y-tube olfactometer to synthetic multistriatin. Weevil behavior was recorded as: pheromone choice, pentane (control) choice, both arms visited, no choice and non-responding (inactive weevils). According to the weevils that made a choice, differences between the number of weevils that chose pheromone or pentane indicated by *$P \leq 0.1$, ***$P < 0.001$ and not significant (ns, at $P > 0.1$) (Pearson's Chi-square test results in Table 1).

Figure 4. Mean (± SE) number of weevils captured per trap and per day in modified funnel traps deployed in (A) Trial 1 and (B) Trial 2. For each weevil sex, bars labelled with the same letter are not significantly different (ANOVA, LSD test at $P > 0.05$).

Table 1 Response of Female and Male *Diocalandra frumenti* (N = 40) in a Y-tube Olfactometer to Synthetic Multistriatin

dose (μg)[a]	sex[b]	number of weevils[c]		total choice[d]	% response pheromone[e]	% response pentane[e]	statistics[f]	
		pheromone	control				χ^2	P-value
100	F	18	6	24	75.0	8.0	12.00	0.001
100	M	13	18	31	41.9	42.9	1.61	0.155
10	F	28	1	29	96.6	1.0	50.28	< 0.001
10	M	22	4	26	84.6	4.7	24.92	< 0.001
0.1	F	21	2	23	91.3	2.2	31.39	< 0.001
0.1	M	11	6	17	64.7	9.3	2.94	0.085
0.001	F	7	8	15	46.7	17.1	0.13	0.500
0.001	M	8	8	16	50.0	16.0	0.00	0.638

[a] dose of multistriatin tested

[b] weevil sex: males (M), females (F)

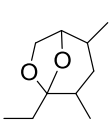
[c] number of weevils that chose either arm: baited with multistriatin (pheromone) or pentane (control), being 40 the total number of weevils of each sex that were employed in these tests

[d] total number of weevils that made a choice (meaning that chose either arm)

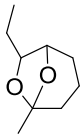
[e] percentage of weevils that chose either arm, based on the number of weevils that made a choice

[f] statistics: Chi-square goodness of fit test.

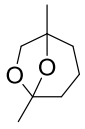
Figure 1



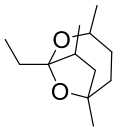
multistriatin, **1**



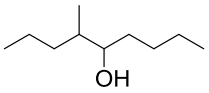
brevicomin, **2**



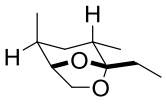
frontalin, **3**



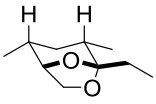
sordidin, **4**



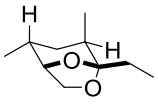
ferrugineol, **5**



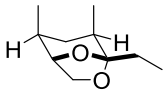
(±)-δ-**1**



(±)-α-**1**



(±)-γ-**1**



(±)-β-**1**

Figure 2

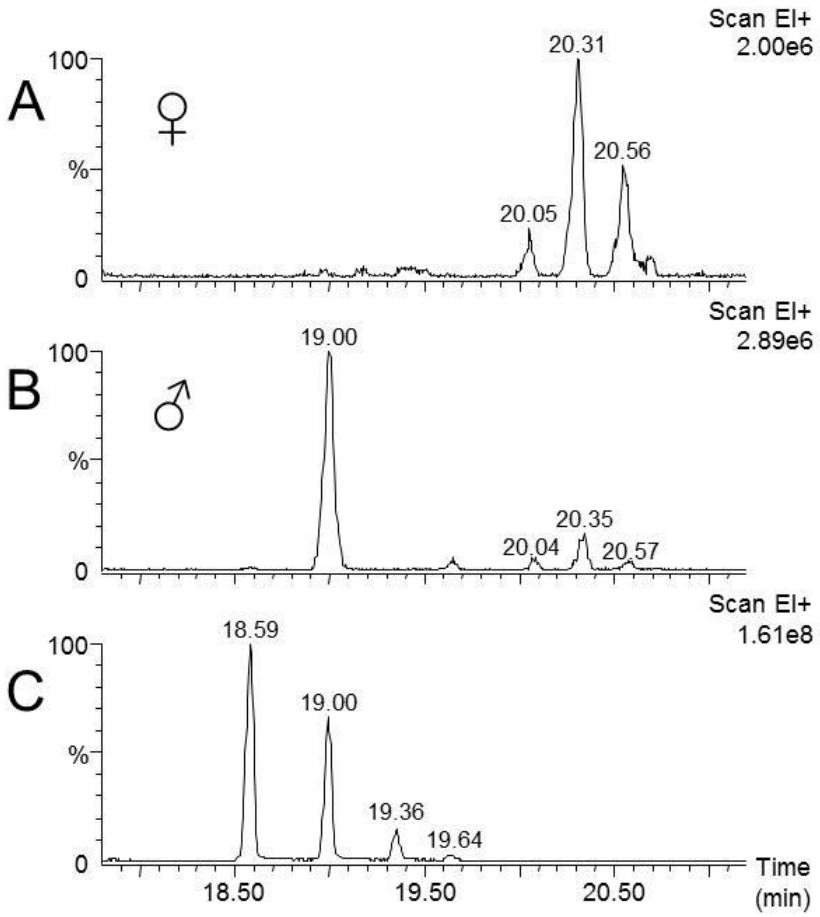

A
Scan EI+
2.00e6
100
%
0
20.31
20.56
20.05
B
Scan EI+
2.89e6
19.00
20.35
20.04
20.57
C
Scan EI+
1.61e8
18.59
19.00
19.36
19.64
18.50
19.50
20.50
Time
(min)

Figure 3

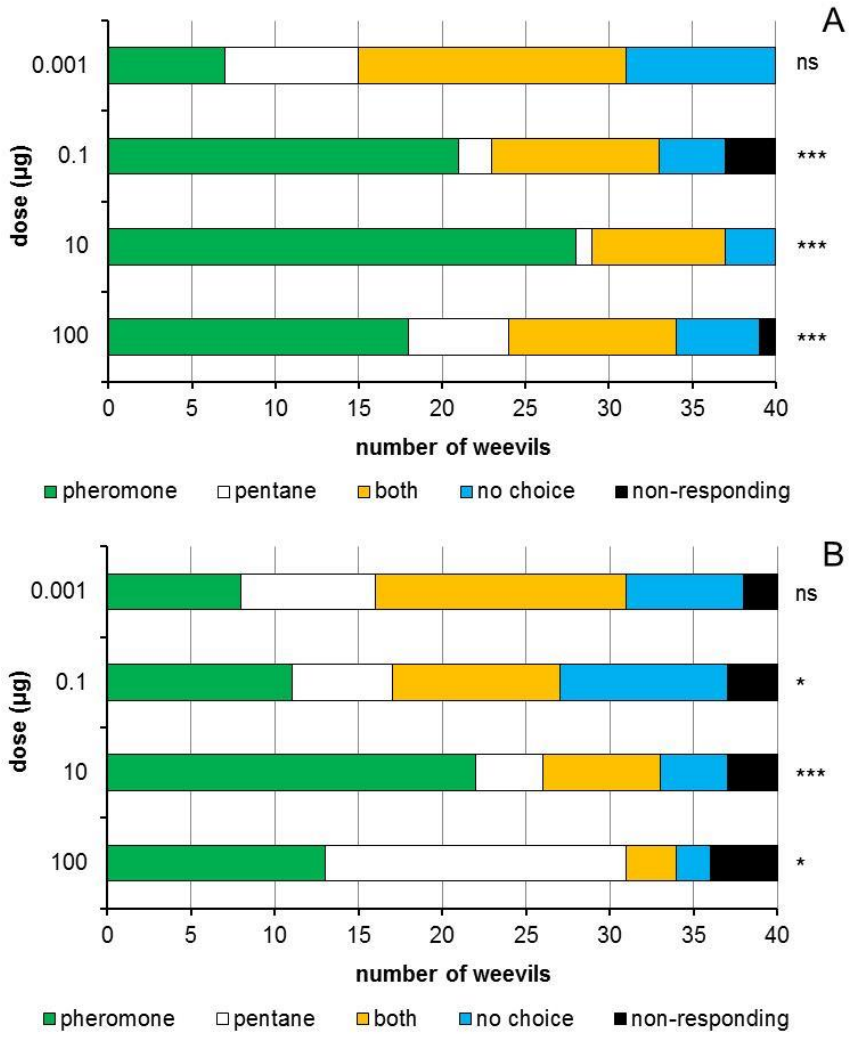
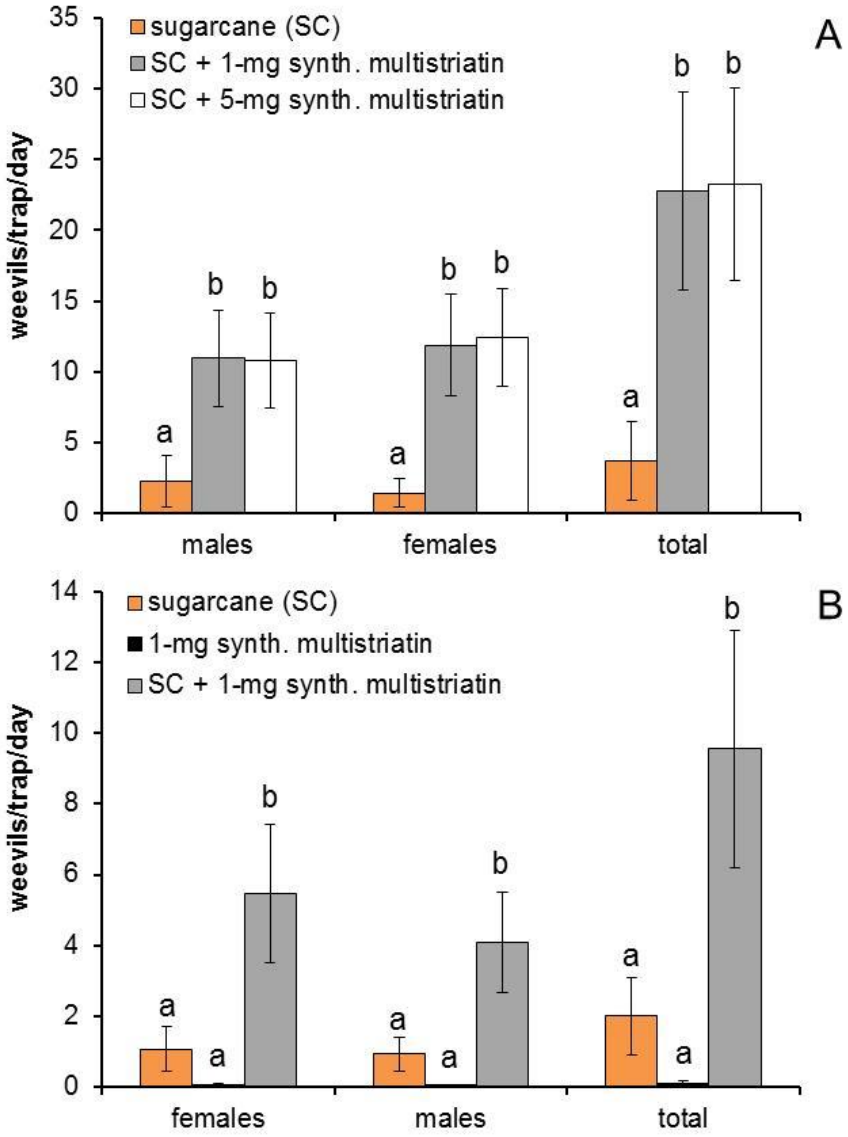

A
0.001
0.1
10
100
dose (µg)
ns
***
***
***
0
5
10
15
20
25
30
35
40
number of weevils
pheromone
pentane
both
no choice
non-responding
B
0.001
0.1
10
100
dose (µg)
ns
*
***
*
0
5
10
15
20
25
30
35
40
number of weevils
pheromone
pentane
both
no choice
non-responding

Figure 4


A
sugarcane (SC)
SC + 1-mg synth. multistriatin
SC + 5-mg synth. multistriatin
weevils/trap/day
35
30
25
20
15
10
5
0
a
b
b
a
b
b
a
b
b
males
females
total
B
sugarcane (SC)
1-mg synth. multistriatin
SC + 1-mg synth. multistriatin
weevils/trap/day
14
12
10
8
6
4
2
0
a
a
b
a
a
b
a
a
b
females
males
total

TOC GRAPHIC