University of São Paulo
"Luiz de Queiroz" College of Agriculture

Studies on the chemical ecology of the wood-boring beetles *Euplatypus parallelus* (Coleoptera: Curculionidae) and *Dinoderus minutus* (Coleoptera: Bostrichidae)

Hugo Leoncini Rainho

Thesis presented to obtain the degree of Doctor in Science. Area: Entomology

Piracicaba
2021

Hugo Leoncini Rainho
Agronomist Engineer

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Advisor:
Prof. Dr. **JOSÉ MAURÍCIO SIMÕES BENTO**

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DEDICATION

This indefeasible work of my life is dedicated to my family, Carlos Alberto Rainho, Suzete Coelho Leoncini Rainho, Heitor Leoncini Rainho, and to my lovely wife, Aline Gastardeli de Oliveira.

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EPIGRAPH

“It is always darkest just before the day dawneth.”

Thomas Fuller *– A Pisgah-Sight of Palestine and the Confines Thereof (1650)*

SUMMARY

RESUMO

Estudo sobre a ecologia química das coleobrocas *Euplatypus parallelus* (Coleoptera: Curculionidae) e *Dinoderus minutus* (Coleoptera: Bostrichidae)

O besouro-da-ambrosia, *Euplatypus parallelus* (Fabricius) (Coleoptera: Curculionidae: Platypodinae) e a broca-do-bambu, *Dinoderus minutus* (Fabricius) (Coleoptera: Bostrichidae: Dinoderinae), estão entre as pragas mais cosmopolitas e economicamente importantes das suas respectivas famílias. Apesar da importância econômica destes besouros, poucas estratégias eficientes e ecologicamente seguras para o seu controle estão disponíveis. Semioquímicos, incluindo compostos atraentes e repelentes, têm sido utilizados para o Manejo Integrado de Pragas (MIP) das famílias Curculionidae e Bostrichidae em todo o mundo. Atraentes feromonais ou voláteis de plantas (cairomônios) não têm sido investigados para *E. parallelus* e *D. minutus*. Na presente tese, um feromônio sexual putativo e atraentes voláteis de plantas para *E. parallelus* e *D. minutus* foram identificados e validados em experimentos de campo. Essa tese foi dividida em dois capítulos. O **Capítulo 1** apresenta a identificação e avaliação de uma mistura de compostos voláteis sexo-específicos, produzidos por machos de *E. parallelus*, combinada ou não ao etanol, um composto presumidamente produzido por plantas hospedeiras desse besouro. O **Capítulo 2** apresenta a identificação e avaliação de compostos orgânicos voláteis produzidos por plantas de bambu que desempenham uma função na localização hospedeira para machos e fêmeas de *D. minutus*. No **Capítulo 1**, os dados mostraram que: (i) machos de *E. parallelus* produzem cinco compostos sexo-específicos (três álcoois, um monoterpeno álcool e um acetato éster), que constituem um sinal químico (i.e., feromônio sexual putativo) que atua na atração de fêmeas conspecíficas somente quando combinado ao etanol supostamente produzido por plantas hospedeiras em condição ideal para a reprodução dessa coleobroca; (ii) machos de *E. parallelus* são atraídos somente por etanol, que serve como um sinal químico determinante para localização de plantas hospedeiras por machos dessa espécie; (iii) potenciais inimigos naturais de *E. parallelus*, pertencentes ao gênero *Sosylus* (Coleoptera: Bothrideridae), são atraídos pela combinação da mistura dos compostos produzidos por machos de *E. parallelus* com etanol, revelando que estes inimigos naturais utilizam os canais químicos de comunicação da sua presa para localizá-la. No **Capítulo 2**, foi observado que: (i) colmos de plantas de bambu se tornam atrativos e começam a ser broqueados por adultos de *D. minutus* após o corte das plantas, o que está relacionado com os compostos voláteis liberados pelos colmos; (ii) adultos de *D. minutus*, tanto machos quanto fêmeas, são atraídos por uma mistura de compostos liberados pelos colmos cortados de bambu, a qual desempenha uma função na localização de plantas hospedeiras em condição ideal para reprodução dessa espécie. Os semioquímicos identificados são promissores para o monitoramento e controle das coleobrocas *E. parallelus* e *D. minutus*.

Palavras-chave: Besouro da ambrosia; Broca do bambu; Feromônio sexual; Cairomônios; Semioquímicos; Voláteis de planta; Atraentes; Interação inseto-planta; Interação tritrófica; Seleção hospedeira; Ecologia Química

ABSTRACT

Studies on the chemical ecology of the wood-boring beetles *Euplatypus parallelus* (Coleoptera: Curculionidae) and *Dinoderus minutus* (Coleoptera: Bostrichidae)

The ambrosia pinhole borer, *Euplatypus parallelus* (Fabricius) (Coleoptera: Curculionidae: Platypodinae), and the bamboo borer, *Dinoderus minutus* (Fabricius) (Coleoptera: Bostrichidae: Dinoderinae) are among the most invasive and economically important pests of their respective families. Despite these beetles' economic importance, a few efficient and ecologically-safe strategies for their management are available. Semiochemicals, including attractant and repellent compounds, have been applied for the Integrated Pest Management (IPM) of beetles within the families Curculionidae and Bostrichidae worldwide. Attractant pheromones or plant volatiles (kairomones) for *E. parallelus* and *D. minutus* have not been addressed. In this thesis, a putative sex pheromone and attractant volatiles of plants for *E. parallelus* and *D. minutus* were identified and validated by field bioassays. This thesis was divided into two chapters. **Chapter 1** presents the identification and evaluation of a blend of male-specific volatile compounds of *E. parallelus*, combined or not to the ethanol, a compound presumably produced by host plants of this beetle. **Chapter 2** presents the identification and evaluation of volatile organic compounds (VOC's) produced by bamboo plants, which play a role in the host location by adult males and females of *D. minutus*. In **Chapter 1**, the results showed that: (i) males of *E. parallelus* produce five sex-specific compounds (three alcohols, one monoterpene alcohol, and one acetate ester), which consisting of a chemical cue (i.e., a putative sex pheromone) on the attraction of conspecific females only when combined with the ethanol supposedly produced by suitable host trees for the breeding of this wood-boring beetle; (ii) males of *E. parallelus* are attracted only to the ethanol, which serves as a determinative chemical cue for the host-plant location by males of this species; (iii) potential natural enemies of *E. parallelus* belonging to the genus *Sosylus* (Coleoptera: Bothrideridae) are attracted to the combination of the *E. parallelus* male-specific compounds with ethanol, revealing that these natural enemies use the chemical channels of communication of its prey to locate it. In **Chapter 2**, it was observed that: (i) culms of bamboo plants become attractive and start to be drilled by adults of *D. minutus* after cutting the plants, which is related to the volatile compounds released by the culms; (ii) adult males and females of *D. minutus* are attracted to a blend of compounds released by the cut bamboo culms, which plays a role in the location of suitable host plants for the breeding of this species. The identified semiochemicals are promising for the monitoring and control of the wood-boring beetles *E. parallelus* and *D. minutus*.

Keywords: Ambrosia beetle; Bamboo borer; Sex pheromone; Kairomones; Semiochemicals; Plant volatiles; Attractants; Insect-plant interaction; Tritrophic interaction; Host selection; Chemical Ecology

1. GENERAL INTRODUCTION

Beetles of the families Curculionidae and Bostrichidae belong to the most threatening pests of cultivated forests, orchards, stored food, and wood-based products. A few species have quarantine importance and are considered the most economically-destructive pests in the world [1–4].

The Neotropical ambrosia pinhole borer, *Euplatypus parallelus* (Fabricius) (Coleoptera: Curculionidae), is considered both the most invasive and economically noxious species within the subfamily Platypodinae [5]. This polyphagous beetle attacks trees affected by abiotic and/or biotic stressing factors, building galleries throughout the bark and penetrating the xylem deeply [6,7]. Besides, promote the inoculation of beetle-associated fungi to the wood tissues that result in trees' death and reduced tree stands. The fungi inoculated by the beetles cause the staining of the wood, severely compromising its quality. In some cases, beetle-associated pathogenic fungi might be causal agents of trees' die-back wilt disease [7–9].

The bamboo borer, *Dinoderus minutus* (Fabricius) (Coleoptera: Bostrichidae), is the major pest of post-harvested bamboo and its resulting products [10,11]. This beetle is present in almost all of the world continents and has undeniable quarantine importance [3,11]. *Dinoderus minutus* is primarily associated with hosting plants of bamboo (Poaceae: Bambusoideae) [10,12]. Adult beetles attack only cut, broken, or felled culms of bamboo plants. The economic damage is due to the holes and galleries and continuous feeding of adults and larvae throughout the parenchyma tissues of bamboo culms and bamboo-derived products [10].

Wood-boring beetles are remarkably referred to as difficult-to-control pests [13,14]. When available, semiochemical-based methods represent efficient and economically safe strategies for controlling such insect pests, including monitoring, mass trapping, and push-pull [14]. These methods require the availability of attractant lures and/or repellents, generally developed from synthetic insect pheromones and volatile organic compounds produced by plants [14]. Attractant pheromones have been successfully determined and applied for the management of the major pests among the ambrosia pinhole borers (Curculionidae: Platypodinae) [15–19] and the powderpost beetles (Bostrichidae) [20–22]. In some cases, lures' attractant effect can be enhanced by the combination of insect pheromones with plant-derived kairomones [23,24].

Despite its economic importance, insect pheromones and plant kairomones have not been addressed for the invasive wood-boring beetles *E. parallelus* and *D. minutus*. Based on the potential of the use of semiochemicals for the management of these wood-boring beetles, the objectives of this thesis for both *E. parallelus* and *D. minutus* were: (i) to identify potential attractant pheromones; (ii) to identify potential plant kairomones; (iii) to demonstrate the biological activity of the candidate semiochemicals by field bioassays; (iv) to verify possible attractant effects of the candidate semiochemicals on the natural enemies of these phytophagous insect-pests by field bioassays.

The results of **Chapter 1** show that the males of *E. parallelus* produce five sex-specific volatile organic compounds. Males of *E. parallelus* are attracted to the ethanol to locate suitable host trees and, subsequently, produce the sex-specific volatile compounds (i.e., the putative sex pheromone). A blend

composed of the male-produced volatile compounds is biologically operative for the attraction of conspecific females only when combined with ethanol supposedly produced by suitable host plants to breed the beetles (i.e., physiologically-stressed trees). The chemical cue composed of the plant-produced ethanol + male-specific volatile compounds is critical for conspecific females to locate suitable trees for mating and reproduction. Also, two species of potential natural enemies of *E. parallelus,* belonging to the genus *Sosylus* (Coleoptera: Bothrideridae) were attracted to combining the male-specific volatile compounds with ethanol, revealing that they use the chemical channels of communication of *E. parallelus* to locate their prey. Further studies are necessary to investigate if the presence of all male-specific compounds in a blend is obligatory to trigger the attraction of conspecific females as well as the bothriderid natural enemies.

The results of **Chapter 2** demonstrate that adults of *D. minutus* are attracted and start to build galleries on the bamboo culms after cutting, which is related to volatile emissions of the bamboo culms. Adult males and females of *D. minutus* are attracted to a blend of volatile compounds released by the cut bamboo culms, which plays a role in the location of suitable host plants for the breeding of this species. Therefore, the encounter of both sexes of *D. minutus* for mating occurs in the culms of decaying plants of bamboo essentially mediated by the plant-derived kairomones. The existence of a close-range sex pheromone involved with the location and recognization of sex partners should be considered in further studies.

The potential of the use of the readily-available semiochemicals determined by this study for the management of the *E. parallelus* and *D. minutus* was discussed.

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2. SEMIOCHEMICAL-BASED ATTRACTANT FOR THE AMBROSIA PINHOLE BORER *Euplatypus parallelus*

Hugo L. Rainho*, Weliton D. Silva and José Maurício S. Bento

Department of Entomology and Acarology, University of São Paulo, Piracicaba, SP, Brazil

*Correspondence: Avenida Pádua Dias, 11, University of São Paulo, Piracicaba, SP 13418900, Brazil. E-mail: hugo.lleoncini@gmail.com

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Abstract

A semiochemical-based attractant for *Euplatypus parallelus* was identified and field-tested. Analyses of headspace volatile extracts of conspecific males revealed the presence of 1-hexanol along with lesser amounts of 3-methyl-1-butanol, hexyl acetate, 1-octanol and trans-geraniol, which were not found in equivalent extracts from females. Emission of 1-hexanol coincided with the emergence of adults of both sexes during afternoon hours. A synthetic blend of these compounds, with and without ethanol, was tested in the field. The blend alone attracted a small number of females and no males. Ethanol alone attracted a small number of females (not significantly different from the blend alone) but significantly more males than the blend alone. More females were caught with the blend combined with ethanol than the combined catch of either attractant alone, suggesting a synergistic interaction. Attraction of males appeared to be a response to ethanol alone. During the trials, two potential natural enemies of *E. parallelus* were caught, indicating that they might be eavesdropping on the semiochemical channels of their prey. Traps containing the male-specific volatile compounds combined with ethanol could be applied as an effective attractant for detection and monitoring of *E. parallelus* as well as for recruitment of its natural enemies.

Keywords: ambrosia beetle; chemical ecology; pheromone; traps; quarantine species; monitoring; Platypodinae; Bothrideridae

2.1. Introduction

The Neotropical ambrosia pinhole borer, *Euplatypus parallelus* (Fabricius) (Coleoptera: Curculionidae: Platypodinae), is one of the most important invasive forest pests worldwide, causing damage in natural and managed forests in over 50 countries [1–3]. This beetle is highly polyphagous, attacking conifers and broadleaf trees of over 80 species from ~25 botanical families [2–4]. Adult *E. parallelus* damage trees by boring deep galleries into the wood, inside of which they inoculate fungal symbionts that create a substrate for their larvae to feed [2,5]. In addition to the damage by the beetles, staining of the woody tissues by the associated fungi compromises the wood quality specially for furniture and veneer production [2,6].

Outbreaks of *E. parallelus* are commonly associated with massive attacks on trees stressed by biotic or abiotic factors, such as damage by other insects, phytopathogens, storms, drought, fire, and forestry management practices [2,7–14]. The attacks compromise the physiology of trees, making them vulnerable to infection by phytopathogens that may result in high mortality in tree stands [7–9,12]. In Brazil, outbreaks of *E. parallelus* have been reported in commercial plantations of *Pinus* [10], *Eucalyptus* [11], *Hevea brasiliensis* (Willd. ex A. Juss.) Müll.Arg. [15], and *Khaya senegalensis* (Desv.) A. Juss. (present study, see Supplementary Material).

Attraction of adult platypodine beetles to stressed trees is mediated by volatile organic compounds (VOCs) [16,17]. Ethanol is released in large amounts by stressed trees [18–20], and platypodine beetles use this VOC as a chemical cue to locate their host [21,22]. In this case, adult males appear to locate and initiate colonization of a suitable host tree. After initiating a gallery, males emit a pheromone to attract conspecific females for mating and reproduction [5].

To our knowledge, male-produced attractant pheromones in platypodine beetles have been identified for five species: *Megaplatypus mutatus* (Chapuis) [23,24], *Myoplatypus flavicornis* (Fabricius) [25], *Platypus cylindrus* (Fabricius) [26], *Platypus koryoensis* (Murayama) [27], and *Platypus quercivorus* (Murayama) [28]. The pheromone of *E. parallelus* has not hitherto been addressed. Despite evidence of attraction of adult *E. parallelus* to ethanol-baited traps [29], there is no further information, either on the efficacy of this alcohol compared to other attractants or controls or on the sex ratio of attracted beetles.

Traps containing semiochemical blends (*e.g.*, pheromones and kairomones) have been applied with success in surveillance programs for native and exotic forest pests [30]. Our objective was to identify a semiochemical-based attractant for *E. parallelus* that could be incorporated into traps for early detection or delineation of the geographical spread of this invasive species.

2.2. Materials and Methods

2.2.1. Source of Chemicals

The authentic standards 3-methyl-1-butanol (purity ≥99%, CAS No. 123-51-3), 1- hexanol (≥99%, 111-27-3), hexyl acetate (99%, 142-92-7), 1-octanol (≥99%, 111-87-5), and *trans*-3,6-dimethyl-2,6-octadien-1-ol (geraniol; 98%, 106-24-1) were purchased from Sigma-Aldrich (Darmstadt, Hessen, Germany).

2.2.2. Source of Beetles

Adult males and females of *E. parallelus* were obtained from infested trees of African mahogany, *Khaya senegalensis* (Desv.) A. Juss. (Meliaceae), from a 15-year-old plantation (~40 ha) located in the city of Inocência, in the Brazilian state of Mato Grosso do Sul (19°13'19" S 52°09'31" W) on 30 October 2017. This plantation suffered a serious outbreak of *E. parallelus* in that year, which resulted in the typical symptoms of dieback wilt syndrome in many trees (Figure S1; Supplementary Material). Infested trees were sawn into logs (50 cm long × 25 cm diameter) and sent by courier to the Laboratory of Chemical Ecology and Insect Behavior, University of São Paulo, Piracicaba, SP (~800 km from Inocência). The logs were housed in two black plastic containers (89 cm long × 56 cm width × 48.5 cm height), and each container received three logs. Because adults of *E. parallelus* are positively phototactic, the newly emerged beetles were recovered from translucent bottles attached to the bottoms of the containers. The material was kept in a greenhouse with no control of environmental temperature and humidity. One of the containers was serviced daily from 16:00 to 18:00 h and the emerged beetles were used for headspace volatile collections. To determine the circadian rhythm of emergence of *E. parallelus*, the other container was checked for emerged beetles every two hours from 06:00–20:00 h and once from 20:00– 06:00 h over 19 days in November 2017.

In the laboratory, adults of *E. parallelus* were separated by sex under a stereomicroscope, based on morphological features described by Thube et al. [31]. Males have prominent spinelike projections on the declivity of the elytra, the striae of elytra are deeply impressed, and the body length is slightly shorter than that of females, whereas females lack elytral projections and impressed striae. Beetles were placed in groups of 10–20 individuals of the same sex in 50 mL Falcon tubes containing paper strips for perching and kept under controlled environmental conditions (25 ± 2 °C, 60 ± 10% RH, 12:12 L:D and 5000 lux) ~20 h prior to the headspace volatile collections.

2.2.3. Collection of Beetle-Produced Volatile Compounds

Headspace volatiles were collected from groups of 10–75 beetles (2–3 days old) of the same sex in cylindrical glass 500-mL chambers. The inner surfaces of the chambers were lined with paper towels to provide a surface for perching. Volatiles were collected in glass pipettes (8.5 cm long × 0.5 cm i.d.) containing 150 mg of 80/100 mesh HayeSep® Q adsorbent (Supelco, Bellefonte, PA, USA) held in

place with glass wool plugs. Collectors were connected to outlets of chambers with screw caps fitted with PTFE ferrules. Activated-charcoal-filtered air was pushed through the chamber at constant flow of ~150 mL/min. Groups of beetles were continuously aerated for 48 h and then discarded. Chambers containing only paper towels were aerated in parallel to monitor system contaminants. Volatiles were eluted from collectors with three successive aliquots of 500 µL of doubledistilled hexane into 2-mL silanized amber glass vials, which were stored at −30 °C. Nine aeration extracts were obtained from each sex. The resulting extracts were not concentrated for the analyses.

2.2.4. Identification of Beetle-Produced Volatile Compounds

Headspace volatile extracts were initially analyzed using gas chromatography with flame ionization detection (GC–FID) to track sex-specific compounds, i.e., the potential attractant pheromone candidates. Two-microliter aliquots were injected into a GC-2010 gas chromatograph (Shimadzu Corp., Kyoto, Japan) fitted with a Rtx-1 capillary column (30 m × 0.25 mm i.d. × 0.25 µm film; Restek, Bellefonte, PA, USA). Injections were made splitless (purge valve off for 1 min) with injector port set at 250 °C and helium carrier gas at a linear velocity of 30 cm/s. The GC oven was programmed at 35 °C (held 1 min), increased to 40 °C at 2 °C/min (held 1 min), and then increased to 250 °C at 10 °C/min (held 10 min).

Extracts containing compounds collected from adult males of *E. parallelus* were analyzed using gas chromatography–mass spectrometry with a Shimadzu QP2010 Ultra GCMS (Shimadzu Corp., Kyoto, Japan) fitted with a Rtx-1MS nonpolar column (30 m × 0.25 mm × 25 µm film; Restek, Bellefonte, PA, USA). One microliter was injected splitless with an injector and GC oven temperatures were set as described above with helium carrier gas at 44 cm/s and 80.8 kPa inlet pressure. Ion source and quadrupole were set at 250 °C. Mass spectra were recorded in electron impact mode (70 eV) from *m/z* 35–260 amu, with 4-min solvent delay. The candidate pheromone compounds were identified by retention indexes and mass spectra similarity with Library NIST 11 and confirmed by co-injection with authentic synthetic standards. Retention indexes were calculated by comparison with a blend of C_5-C_{30} straight-chain alkane standards (Sigma-Aldrich, Darmstadt, Hessen, Germany).

2.2.5. Emission of the Major Male-Specific Volatile Compound Over Time

We ran an extra set of aerations of adult males of *E. parallelus* to determine the time of emission of the major sex-specific volatile compound. Groups of 12 adult males (n = 6 replicate groups) were aerated from 06:00 to 22:00 h and volatiles were eluted from collectors at every 4-h interval. Elutions were done with two successive aliquots of 150 µL of double-distilled hexane spiked with 1 ng/µL of nonyl acetate as internal standard. Because 1-hexanol was the major and most frequent component in male-specific volatile extracts (see Results), the quantifications were made on this compound. The results were expressed as nanograms of 1-hexanol per male per 4-h interval.

2.2.6. Field Bioassay of Sythetic Male-Specific Volatile Compounds

The synthetic blend of male-specific volatile compounds of *E. parallelus* (see Results) were field-tested in the same African mahogany plantation in Inocência (see above) from 13 October 2018 to 1 November 2018. We used custom-made cross-vane traps (translucent polyethylene terephthalate glycol panels; 24 cm high × 19.5 cm width). Translucent traps have shown excellent results for capture of other platypodine beetles—for example, *M. mutatus* in South America [32]. Collection jars (500 mL) were attached to the trap basins and filled with 250 mL of an aqueous solution of polypropylene glycol (20%) to kill and preserve the captured beetles. Traps were suspended from inverted L-shaped hangers of PVC pipe (1.8 m × 2.5 cm i.d.), which were mounted on 1-m reinforcing steel bars hammered halfway into the ground, so the trap base was at ~1.2 m high. Lures consisted of clear polyethylene press-seal sachets (5 cm width × 7 cm height, 80 µm wall thickness, Daiso Ind. Co., Hiroshima, Japan) containing a roll of dental cotton loaded with 1-mL solution of a blend of male-produced volatiles in isopropanol. Lures were hung in the central open slot of the traps with pieces of plastic-coated wire.

The amount of each compound used per lure was based on the natural proportion emitted by adult males (see Results). The release ratio of the major component was similar to those described in previous studies with other platypodine species (i.e., ~28 mg/day) [32–34]. Because it was proved that adult *E. parallelus* are attracted to ethanol [29], we included a treatment lure composed of a 30-mL polyethylene flask filled with 99.5% ethanol. The flask was fitted with a 7-cm-long cotton-string wick to provide a high ethanol release rate (~2 g/day), which is necessary to trigger the attraction of ambrosia beetle species [21,35]. Thus, the field bioassay comprised the following treatments: (1) MSV (male-specific volatiles) = blend of 3-methyl-1-butanol (1.5 mg), 1-hexanol (50 mg), hexyl acetate (2 mg), 1-octanol (3 mg), and geraniol (0.5 mg); (2) ethanol (EtOH; 20 mL); (3) MSV + EtOH; and (4) control (1 mL of neat isopropanol). Isopropanol was chosen because we have used it as a standard solvent for coleopteran pheromones [36] and it appears not to affect the attraction of beetles at the concentrations used here.

Treatments were assigned randomly to traps in five blocks, and each block contained one trap for each treatment. Traps were placed 30 m apart and blocks were spaced 50 m from each other. Traps were checked daily for captured beetles, at which time treatments were changed one position within blocks to control for positional effects. MSV lures were replaced every two days to prevent complete depletion of the minor components, and flasks were refilled with ethanol when the volume was depleted by 50%. Captured beetles were kept in Falcon tubes filled with 70% ethanol until identification in the laboratory. Beetles were collected under ICMBio permit #60705-2, issued by the Brazilian Ministry of the Environment. Voucher specimens of *E. parallelus* were deposited in the “Luiz de Queiroz” Museum of Entomology, Department of Entomology and Acarology (USP/ESALQ), Piracicaba, SP, Brazil, under register codes ESALQENT000053 to ESALQENT000062.

2.2.7. Statistical Analysis

Differences between treatment means in the number of beetles caught were tested individually for species (represented by at least 10 specimens), using the nonparametric Friedman's test (PROC FREQ, option cmH) [37] because the assumptions of ANOVA were violated by heteroscedasticity [38]. Replicates were considered as a block and collection date and replicates with zero captures (due, for example, to inclement weather) were omitted from the analysis. In recognition of the multiple statistical tests of treatment effects, significance level was adjusted by the number of species included in the analyses (i.e., $\alpha = 0.017$, $n = 3$ independent analyses), according to the Bonferroni procedure [39]. Pairs of means were compared using the Ryan-Einot-Gabriel-Welsch Q multiple range test, which controls the Type I experimentwise error rate [37]. The sex ratio of adult *E. parallelus* emerged from African mahogany logs and caught with traps baited with the optimal attractant was compared to a nominal ratio of 0.5 with 95% Clopper–Pearson exact confidence intervals at 5% probability [40].

2.3. Results

2.3.1. Identification of Beetle-Produced Volatile Compounds

Headspace volatile extracts of adult males of *E. parallelus* showed five peaks that were absent from equivalent extracts of conspecific females (Figure 1) and controls. Their identities corresponded to the following compounds: 3-methyl-1-butanol (Retention Index on Rtx-1 column = 702), 1-hexanol (RI = 820), hexyl acetate (RI = 996), 1-octanol (RI = 1055), and geraniol (RI = 1233), which were produced by beetles in ratios (mean ± SD, $n = 3$ aeration extracts) of 2.6 ± 0.3:100:2.9 ± 0.5:4.3 ± 2.2:1.6 ± 0.8, respectively. Only 1-hexanol and hexyl acetate were found in 100% of male extracts; all other male-specific compounds were found in 75% of the extracts.

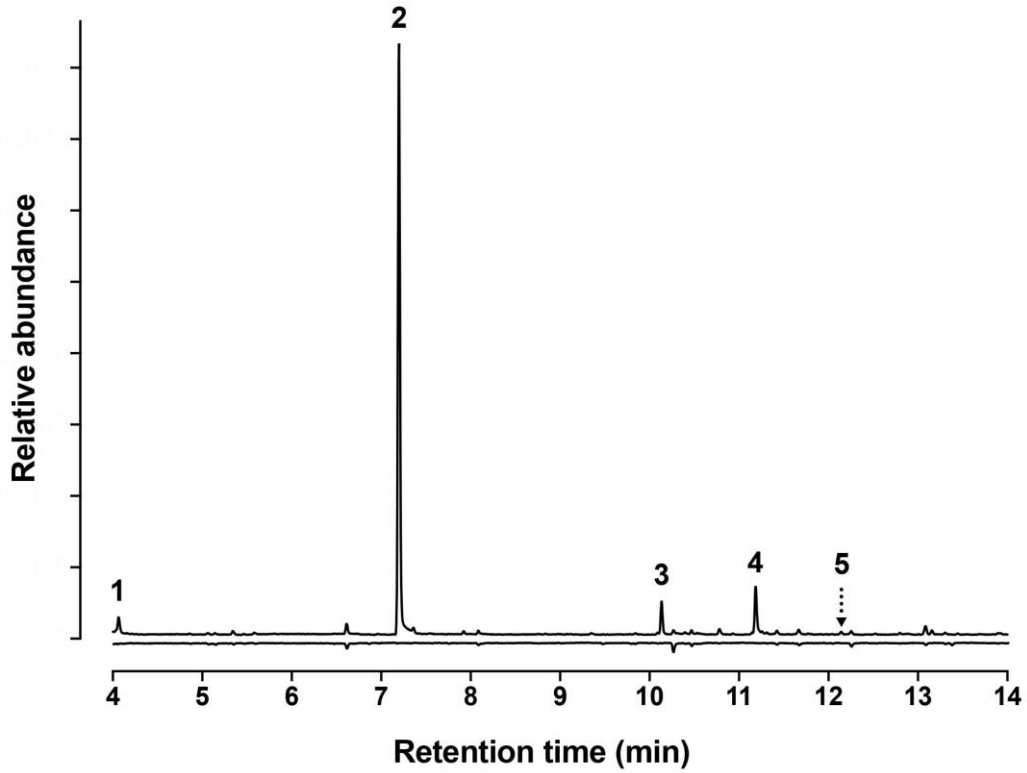

Figure 1. Representative total ion chromatograms of headspace volatile extracts from adult males (top trace) and females (bottom, inverted trace) of *Euplatypus parallelus*. Numbers on peaks represent the male-specific volatile compounds: 1 = 3-methyl-1-butanol; 2 = 1-hexanol; 3 = hexyl acetate; 4 = 1-octanol; and 5 = geraniol.

2.3.2. Circadian Rhythm of Adult Emergence

A total of 191 female and 200 male adults of *E. parallelus* emerged from mahogany logs during 19 days of evaluation. The sex ratio of emerged beetles did not differ significantly from an expected ratio of 0.5 (48.9% females; Clopper–Pearson exact confidence intervals: 0.438–0.4539, p = 0.649). Emergences began in early afternoon with adult males, but a prominent peak of emergence including both sexes occurred between 14:00 and 18:00 h. After that time, emergences declined dramatically. No beetles emerged from 20:00 h until next afternoon (Figure 2).

A total of 46 adults of *Sosylus* cf. *cursorius* (Pascoe) and *Sosylus squirei* (Pascoe) (Coleoptera: Bothrideridae) emerged from African mahogany logs during the 19-day collection.

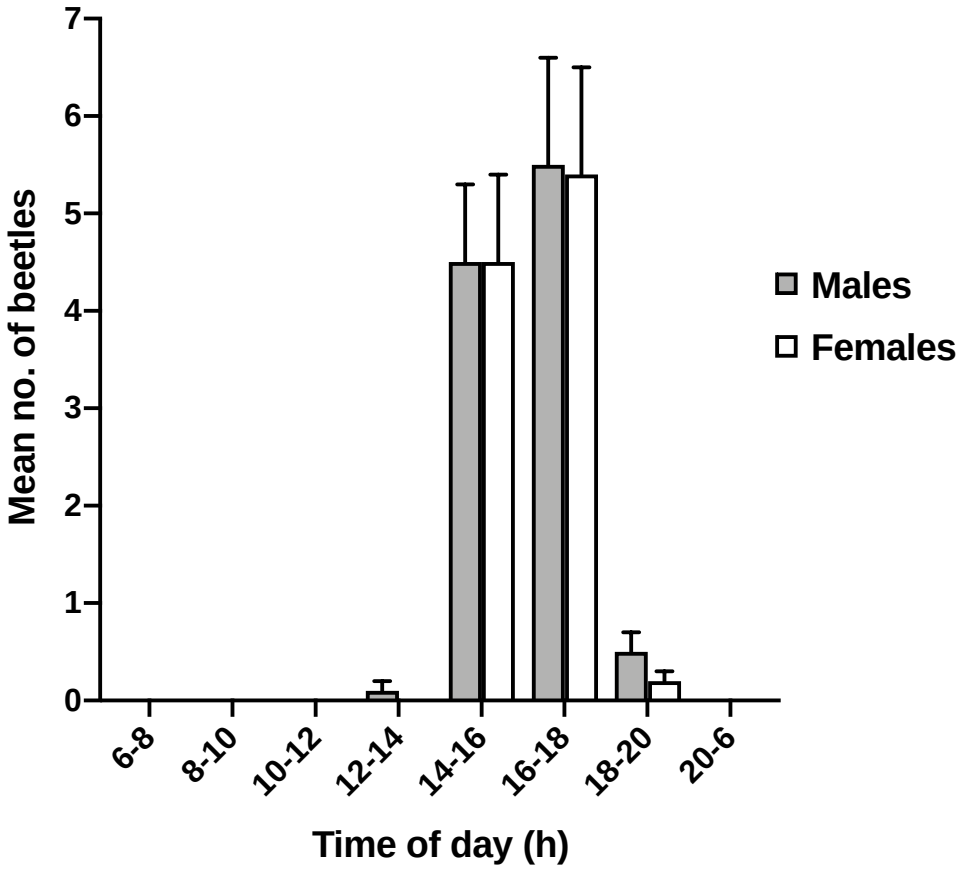

Figure 2. Mean (± SE) numbers of adult females and males of *Euplatypus parallelus* emerged from logs of *Khaya senegalensis* over different times of day during 19 days of evaluation.

2.3.3. Emission of the Major Male-Specific Volatile Compound Over Time

1-Hexanol was emitted by adult males of *E. parallelus* in all time intervals evaluated. Peak emission occurred from 14:00 to 18:00 h (Figure 3), corresponding with peak adult emergence (see above).

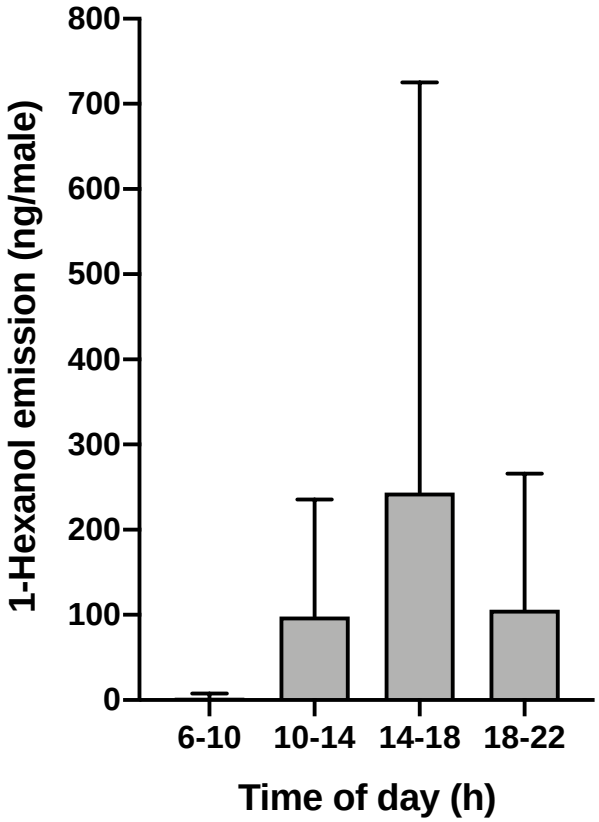

Figure 3. Mean (± SD) amount of 1-hexanol emitted per male of *Euplatypus parallelus* in every 4-h interval from 06:00–22:00 h.

2.3.4. Field Bioassay of Synthetic Male-Specific Volatile Compounds

Overall, 302 adult *E. parallelus* (194 males and 108 females) were caught during the field bioassay to test the reconstructed blend of male-specific volatile compounds. Adult females were significantly attracted to traps containing the combination male-specific volatiles (MSV) + ethanol (mean ± SE of 1.7 ± 0.2 beetles/replicate) (Figure 4). A small number of females, not significantly different from zero, were collected in traps containing the MSV alone or ethanol alone. Adult males were attracted equally to the MSV + ethanol (1.9 ± 0.3) and ethanol alone (1.2 ± 0.3). No males were captured in traps baited with the MSV in the absence of ethanol (Figure 4). The sex ratio of beetles caught with the MSV + ethanol was male-biased (42.4% females; Clopper–Pearson exact confidence intervals: 0.355–0.495, $p = 0.029$). No other platypodine species was captured during the bioassay.

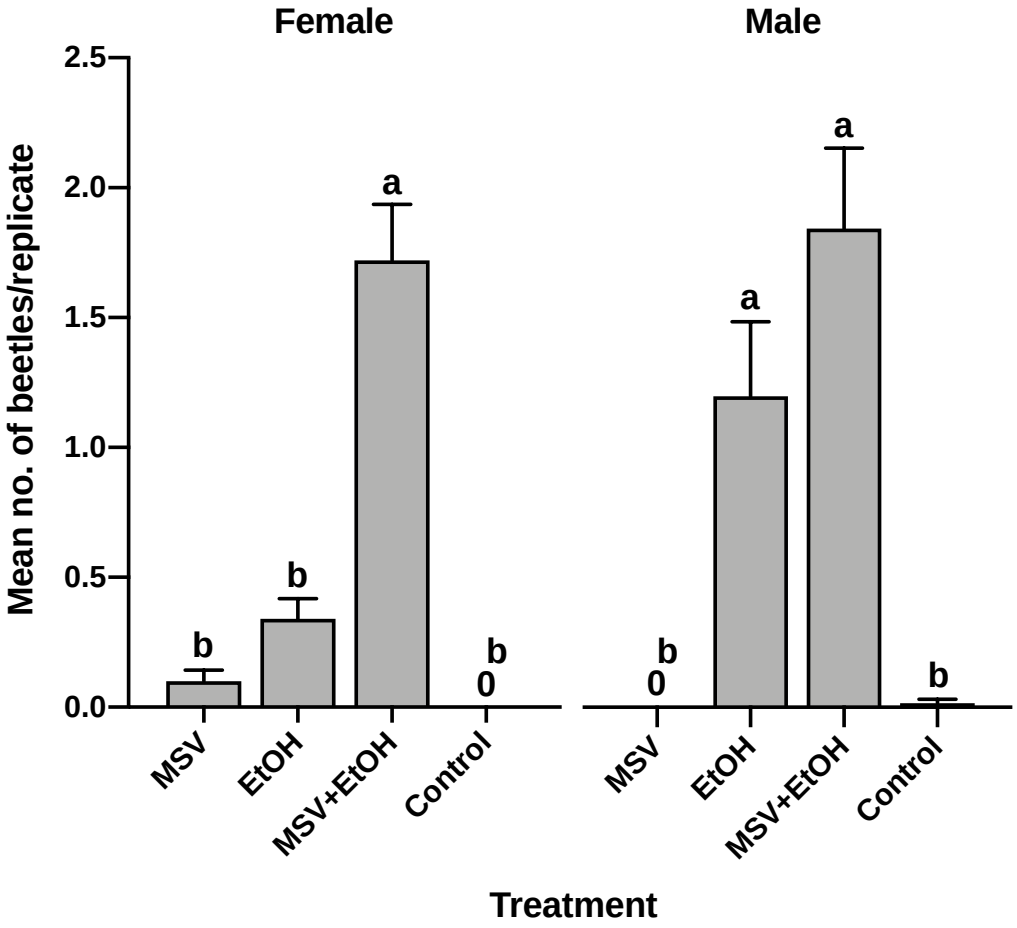

Figure 4. Mean (± SE) numbers of adult females and males of *Euplatypus parallelus* caught with traps containing synthetic male-specific volatile compounds in combination or not with ethanol. Treatment abbreviations: MSV = male-specific volatiles, i.e., blend of 3-methyl-1-butanol, 1-hexanol, hexyl acetate, 1-octanol, and geraniol in the same ratio produced by conspecific males; EtOH = 99.5% ethanol; control = neat isopropanol. Type of attractant lure significantly affected the trap catch of females ($Q_{3,200}$ = 101.4, p < 0.0001) and males ($Q_{3,252}$ = 133.6, p < 0.0001). Means followed by same letter within a panel are not significantly different according to the Ryan-Einot-Gabriel-Welsch Q multiple range test at 5% probability.

Adults of the two bothriderid beetle species that emerged from the mahogany logs were also caught in the treatment traps, i.e., 18 adults (sexes combined) of *S*. cf. *cursorius* and 22 adults of *S. squirei*. Adult *S*. cf. *cursorius* were significantly more attracted to the MSV + ethanol compared to other treatments (Figure 5), whereas adult *S. squirei* were attracted in significant numbers to the MSV + ethanol than to ethanol alone and the control, but the combination did not differ statistically from MSV alone (Figure 5).

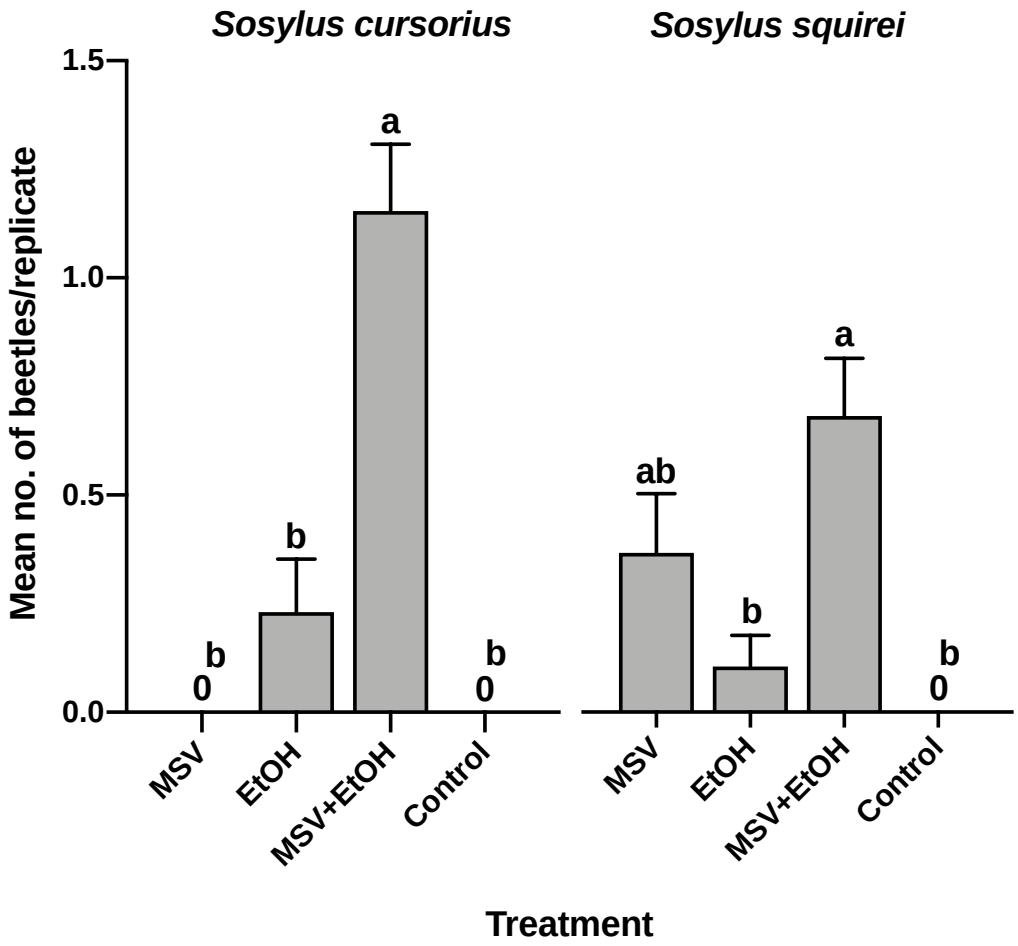

Figure 5. Mean (± SE) numbers of adults of *Sosylus* cf. *cursorius* and *Sosylus squirei* captured with traps containing synthetic male-specific volatile compounds of *Euplatypus parallelus*, in combination or not with ethanol. Treatment abbreviations: MSV = male-specific volatiles of *E. parallelus*, i.e., blend of 3-methyl-1-butanol, 1-hexanol, hexyl acetate, 1-octanol, and geraniol in the same ratio produced by conspecific males; EtOH = 99.5% ethanol; control = neat isopropanol. Type of attractant lure significantly affected the trap catch of *S*. cf. *cursorius* ($Q_{3,52}$ = 36.1, p < 0.0001) and S. squirei ($Q_{3,76}$ = 22.3, p < 0.0001). Means followed by same letter within a panel are not significantly different according to the Ryan-Einot-GabrielWelsch Q multiple range test at 5% probability.

2.4. Discussion

Adult male *E. parallelus* produced five sex-specific volatile compounds, including 1- hexanol and smaller amounts of 3-methyl-1-butanol, hexyl acetate, 1-octanol, and transgeraniol. Some of these compounds have been reported as male-produced pheromone components in other platypodine species, *e.g.*, 1-hexanol and 3-methyl-1-butanol in *M. flavicornis* [25], 1-hexanol in *P. cylindrus* [26], and trans-geraniol in *P. koryoensis* [27].

During the field bioassay, traps containing the synthetic blend of male-produced volatile compounds did not attract adult female *E. parallelus*, equivalent to the catch of traps baited with ethanol or isopropanol (a negative control). Significantly more females were caught in traps baited with the male-specific volatiles + ethanol. Males were not attracted to the male-specific volatiles alone, but they were significantly attracted to ethanol alone. The catch of males did not increase in traps containing ethanol combined with the male-specific volatiles. These results suggest that the male-specific volatiles

comprise the putative sex-pheromone components of *E. parallelus* that attract conspecific females, but this attraction functions only in the presence of ethanol that is presumably produced by trees that have been compromised by environmental stress factors. Synergism implies that the response of insects to the mixture of pheromone and plant volatiles is greater than the sum of the responses to the individual components [41]. This phenomenon has been well documented in Lepidoptera for sex pheromones and in Coleoptera (Curculionidae) for aggregation pheromones [41]. Synergism between insect-produced volatile pheromones and host-produced ethanol is a common feature of the life history of several ambrosia beetle species that colonize stressed trees [42].

Stressed trees attract ambrosia beetles because they release large amounts of ethanol in contrast to healthy trees [18,20]. Ethanol release reflects the suitability of the host for cultivation of fungal symbionts for feeding the beetles' larvae [21,22]. Trees can be stressed by a range of biotic and abiotic factors, including attack by other insects, phytopathogens, storms, fire, drought, flooding, and forestry practices that injure woody tissues and induce metabolic production of ethanol [18,20]. In smaller amounts, ethanol can also be released by fungal fermentation of infected woody tissues [22,43,44].

The putative male-produced sex pheromone combined with ethanol is a critical component in the reproductive behavior of *E. parallelus*. Ethanol is a kairomone that lures adult males to a suitable host tree, in which they initiate galleries and emit a sex pheromone to call in females. Ethanol combined with the sex pheromone guides conspecific females to the host tree for mating and reproduction. This scenario seems to be true for other platypodine species, where adult males are responsible for seeking suitable host trees and subsequently produce attractant pheromones [5].

The sex ratio of adult *E. parallelus* attracted by the blend of male-specific volatiles + ethanol was male-biased, whereas the sex ratio of conspecifics emerged from mahogany logs was 1:1. However, further independent sampling of the actual sex ratio in the mahogany plantation during the course of bioassays should be undertaken to determine whether males are more attracted to this blend than are females.

In addition to these biological data, we found that the peak in emission of the major male-produced volatile compound (1-hexanol) of *E. parallelus* lasts from early afternoon until sunset, which corresponds to the period of greatest emergence of conspecific adults. This diurnal activity rhythm appears to occur in other platypodines—for example, *M. mutatus* [24], *M. flavicornis* [45], and *P. quercivorus* [46].

During the field trial, adults of *S*. cf. *cursorius* and *S. squirei* were attracted to some treatments. These species belong to the coleopteran family Bothrideridae, subfamily Bothriderinae, tribe Deretraphrini [47,48]. Adults of both species were attracted mainly to traps containing the male-specific volatiles of *E. parallelus* + ethanol. Adults of these species were also recovered from the African mahogany logs evaluated. Based on these results, *S*. cf. *cursorius* and *S. squirei* can be considered potential natural enemies of *E. parallelus*, which agrees with previous studies that report other species of the genus as predators and ectoparasitoids of platypodine beetles [49–51]. Adult bothriderids may be eavesdropping on the semiochemical channels of *E. parallelus* to locate their prey.

The increasing global trade of goods and movement of people have contributed to the spread of non-native and invasive insect species, especially forest pests [52]. Semiochemical-based traps have become important for early detection and monitoring of these invaders wherever they occur [30]. The inexpensive, readily available attractant semiochemicals identified here have good potential for use in surveillance programs of *E. parallelus*. Lures containing the male-specific volatile compounds of *E. parallelus* + ethanol could be incorporated into traps for early detection of this species in ports of entry, as well as for delineation of geographical spread and for monitoring in cultivated forests. Moreover, semiochemical-based attractant lures could be deployed in forest plantations to attract female beetles to kill stations (mass trapping), to nonhost (decoy) tree species, as a mating disruptor, or to recruit natural enemies for biological control of this important platypodine species.

We have not determined if all five volatile compounds isolated from male *E. parallelus* are necessary for attraction of females. Furthermore, other volatile organic compounds emitted by suitable host trees, which may enhance the attraction of adult beetles to ethanol, and the optimal release rate of compounds in the lures merit further investigation.

2.5. Conclusions

Adult male *E. parallelus* sex-specifically produced 1-hexanol (major), 3-methyl-1- butanol, hexyl acetate, 1-octanol, and *trans*-geraniol. In the field, a synthetic blend of these volatile compounds, in combination with ethanol, attracted conspecific females. Males were attracted equally to ethanol alone or to male-specific volatiles + ethanol. It is unclear if all the blend compounds are necessary and sufficient for the attraction that we observed. Two potential natural enemies of *E. parallelus* were attracted by the combination male-specific volatiles + ethanol blend. Traps containing this semiochemical-based attractant may be useful in surveillance and management programs for this important platypodine species.

Author Contributions

H.L.R., W.D.S., and J.M.S.B. conceived the study; H.L.R. and W.D.S. obtained the volatile extracts and identified the pheromone candidates; H.L.R. carried out the field bioassays and identified the collected specimens; W.D.S. performed the statistical analyses; H.L.R. wrote the manuscript draft; J.M.S.B. and W.D.S. revised and edited the manuscript draft; J.M.S.B. supervised the research and obtained the funding. All authors have read and agreed to the published version of the manuscript.

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Conflicts of Interest

The authors declare no conflicts of interest.

Acknowledgments

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Supplementary Material

Infestation of African Mahogany by *Euplatypus parallelus* in Brazil

An outbreak of *Euplatypus parallelus* was verified in October 2017 in a 40-ha plantation of *Khaya senegalensis* (Desv.) A. Juss. (Meliaceae), located in the city of Inocência, in the Brazilian state of Mato Grosso do Sul (19°13'19"S 52°09'31"W; 532 m altitude). Trees were 15-year-old and had 20-cm diameter at breast height and presented typical symptoms of dieback wilt syndrome (Figure S1a).

In a chronological order, the symptoms initiated with longitudinal fissures along the bark of trunk followed by resin overflow (Figure S1b). Next, bark and surface of xylem presented necrotic lesions; leaves became chlorotic before they abscised from trees. Finally, branches and other tree parts were progressively wilting until the death of the tree.

In addition, many pinholes were observed throughout the bark as well as accumulation of frass on the tree base due the boring activity of adult *E. parallelus*. Beetles' pinholes concentrated in the first 50 cm from the trunk base and they progressively decreased in number until 3 m high. On the wall of the galleries, we noticed a characteristic black-staining caused by some saprophytic fungi, which was likely inoculated by *E. parallelus*.

The symptoms described above were recorded from two mahogany trees, which were monitored from the first symptoms until their death. The death of trees occurred within 10 days, indicating the existence of highly aggressive phytopathological disorder. The association of multiple physiological stressors on mahogany trees from that region (*e.g.*, severe drought and injuries on woody tissues due forestry practices) likely triggered the attack of *E. parallelus* that culminated with the dieback wilt syndrome. Reduction of African mahogany stands in that location due the syndrome has been estimated at ~ 1.3% (i.e., ~ 200 trees), which corresponds to economic losses of US$ 65.573,77 in the Brazilian market [6].

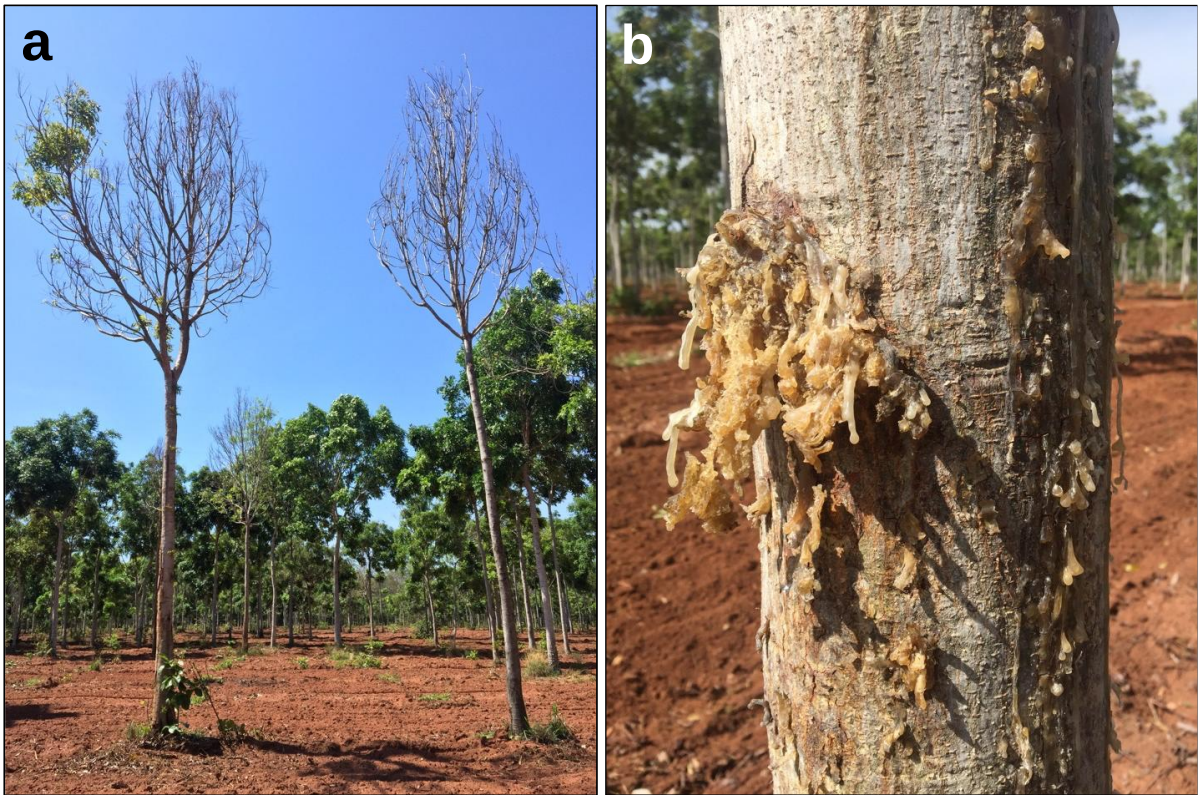

Figure S1. African mahogany, *Khaya senegalensis*, plantation that suffered a serious outbreak of *Euplatypus parallelus* in southwestern Brazil: (**a**) two mahogany trees with symptoms of dieback wilt syndrome; (**b**) mahogany trunk with resinosis. Photos by H.L. Rainho.

3. THE BAMBOO BORER, *Dinoderus minutus* IS ATTRACTED TO A BLEND OF VOLATILE COMPOUNDS PRODUCED BY CULMS OF BAMBOO PLANTS

Hugo L. Rainho*, Weliton D. Silva, Marcoandre Savaris, Felipe G. Gonçalves and José Maurício S. Bento

Department of Entomology and Acarology, University of São Paulo, Piracicaba, SP, Brazil

*Correspondence: Avenida Pádua Dias, 11, University of São Paulo, Piracicaba, SP 13418900, Brazil. E-mail: hugo.lleoncini@gmail.com

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Abstract

The bamboo borer, *Dinoderus minutus* is the major pest of post-harvested bamboo and its products. This cosmopolitan species is a great concern as quarantine pest in tropical and subtropical regions that produce bamboo and its timber products. Semiochemical-based attractants for monitoring and management of this invasive species have not been addressed. Thus, the objective of this study was to identify volatile organic compounds produced by the bamboo culms that comprise an attractant for *D. minutus*. Attraction and boring activity of *D. minutus* to bamboo culms was observed 24 h after cutting the plants. However, the peak of attack intensity by the beetles occurred 35 days after cutting. The release of substantial amounts of five saturated aliphatic aldehydes and a single furan by the cut culms of bamboo was observed at the peak of beetles' attacks. Significant electroantennographic responses of both adult males and females of *D. minutus* were observed to the synthetic hexanal, the major-produced compound by the cut bamboo culms. Synthetic candidate kairomones identified from bamboo culms were tested in field bioassays conducted in Brazil. Traps baited with different blends of the candidate compounds attracted more adult beetles compared to control. Adult males and females were significantly attracted to the bonafide blend, which consists of hexanal + decanal. Thus, this blend is involved with the long-range attraction of adult *D. minutus* to the culms of decaying plants of bamboo.

Keywords: Plant volatiles; Kairomones; Insect-plant interaction; Host-plant location; Primary attraction; Powderpost beetle

3.1. Introduction

The bamboo borer, *Dinoderus minutus* (Fabricius) (Coleoptera: Bostrichidae: Dinoderinae), is the major pest of post-harvested bamboo and its finished products throughout the world [1]. This cosmopolitan species has been reported in 48 countries, belonging to almost all biogeographic regions [2]. The increasing international trade of bamboo-derived products and its timber has raised concerns about the bamboo borer introduction in localities with tropical and subtropical climate and suitable host species of bamboo [3].

Dinoderus minutus has a narrow range of host plants, particularly bamboo species (Poaceae: Bambusoideae) [1,4,5]. The high degree of host-plant specificity of the bamboo borer and the relatively high susceptibility of post-harvested and raw bamboo timber to the attack by the beetle give it overt economic importance [1,6]. Economic damage results from the boring activity and feeding of adults and larvae on the tissues of bamboo culms and their timber products, leading to the destruction of raw materials [1].

Evidence of plant volatile organic compounds (VOC's) mediating the attraction of adult beetles have been reported for the major pests of the stored grain of cereals within the family Bostrichidae, for example, the lesser grain borer *Rhyzopertha dominica* (Fabricius) [7,8] and *Dinoderus bifoveolatus* (Wollaston) [9]. Adult of *D. minutus* attacks only recently felled, broken, or cut bamboo plants' culms [1]. Attacks to live plants of bamboo have not been reported [6]. Thus, we hypothesized that the volatile emissions of decaying bamboo plants' culms play a role in the host-plant location by *D. minutus* adults.

To our knowledge, semiochemical-based attractants (i.e., kairomones and pheromones) for *D. minutus* have not been addressed. This study aimed to identify attractant compounds for adult *D. minutus* among the volatile organic compounds emitted by suitable culms of bamboo plants.

3.2. Materials and Methods

3.2.1. Source of Chemicals

The authentic standards hexanal (purity 98%, CAS No. 66-25-1), heptanal (95%, 111-71-7), octanal (99%, 124-13-0), nonanal (95%, 124-19-6), decanal (≥97%, 112-31-2) and 2-pentylfuran (≥98%, 3777-69-3) were purchased from Sigma-Aldrich (Darmstadt, Hessen, Germany).

3.2.2. Evaluation of the Attack Intensity of Adult D. minutus to Cut Culms of Bamboo Over Time

For this bioassay and volatile collections (for details, see below), we selected the common bamboo, *Bambusa vulgaris* Schrad. (Poaceae: Bambusoideae). This plant material chosen is known as a preferred host-plant species for *D. minutus* [5]. Live plants of *B. vulgaris* were cut during the autumn season because there is evidence that the plants are more susceptible to the attack of *D. minutus* for feeding and reproduction during this period due to the optimal nutritional and decreased secondary

metabolites contents in the culm tissues [10]. A single plant was transversely cut at the base and longitudinally in half. The halves were divided, resulting in 11 “gutter-like” samples (30 cm long × 3 cm radius). For beetles’ colonization, the samples were exposed in a single cluster in the field in Piracicaba, SP, Brazil (22°42’45” S, 47°37’41” W; 548 m altitude), distant ~200 m of plantations of exotic bamboos, *B. vulgaris,* and *Dendrocalamus giganteus* Munro (Poaceae: Bambusoideae) within remnants of Atlantic Rainforest. The number of newly-built gallery holes by adults in each sample was recorded from 1, 7, 14, 21, 28, 35, and 42 days after cutting the culms of bamboo.

3.2.3. Collection of Volatile Organic Compounds Produced by Cut Culms of Bamboo

A single *B. vulgaris* plant was cut following the same previously described protocol (for details, see above). After that, each sample of bamboo was weighted using a precision digital balance. Headspace volatiles were collected from four samples (replicates) of bamboo at the peak of beetles’ infestation (35 days after cutting; for details, see Results). The volatile bamboo compounds were collected by aeration for 24 h in cylindrical glass chambers (41 cm long × 7 cm i.d.) under controlled laboratory conditions (25 ± 2 °C, 60 ± 10% RH, 12:12 L:D and 5000 lux). Volatiles were collected in glass pipettes containing 30 mg of 80/100 mesh HayeSep® Q adsorbent (Supelco, Bellefonte, PA, USA). Collectors were connected to outlets of chambers with screw caps fitted with ferrules of polytetrafluoroethylene (PTFE). Air previously filtered in activated charcoal was pushed through the glass chamber at a constant flow of ~300 mL/min. Empty glass chambers were aerated concomitantly to monitor system contaminants. Volatiles were eluted from collectors with two successive aliquots of 150 µL of double-distilled hexane into 2-mL silanized amber glass vials and stored at −30 °C. The extracts were not concentrated for analysis.

3.2.4. Identification and Quantification of Volatile Organic Compounds Produced by Cut Culms of Bamboo

Headspace extracts were first analyzed by gas chromatography with flame ionization detection (GC-FID). Extract aliquot of 2 µL was injected into a GC-2010 gas chromatograph (Shimadzu Corp., Kyoto, Japan) fitted with a Rtx-1 capillary column (30 m × 0.25 mm i.d. × 0.25 µm film; Restek, Bellefonte, PA, USA). Injections were made in splitless mode with injector port set at 250 °C and helium carrier gas at a linear velocity of 30 cm/s. The GC oven was programmed at 35 °C (held 1 min), increased to 40 °C at 2 °C/min (held 1 min), and then increased to 280 °C at 10 °C/min (held 20 min). For quantification of compounds, 1 µg of nonyl acetate (internal standard) was added to the extracts.

Representative headspace extracts of cut culms of bamboo were analyzed by gas chromatography-mass spectrometry with a Shimadzu QP2010 Ultra GCMS (Shimadzu Corp., Kyoto, Japan) fitted with a Rtx-1MS nonpolar column (30 m × 0.25 mm × 25 µm film; Restek, Bellefonte, PA, USA). Extract aliquot of 1 µL was injected in splitless mode with injector and GC oven temperatures set as described above and helium carrier gas at 44 cm/s and 80.8 kPa inlet pressure. Mass spectra were recorded in electron impact mode (70 eV) from *m/z* 35–260, with 4-min solvent delay. Detected

compounds were identified by the calculation of Kovats retention indexes and mass spectra similarity with the NIST 11 library. Kovats retention indexes were calculated by comparison with a blend of C_5-C_{30} aliphatic alkane standards (Sigma-Aldrich, Darmstadt, Hessen, Germany). The chemical identity of the candidate compounds was confirmed by co-injection with authentic synthetic standards.

3.2.5. Electroantennography (EAG) of the Bamboo Borer D. minutus

The electroantennographic test consisted of a single male or female antenna of *D. minutus*. The antenna was dissected from the head of the beetle and mounted on a Y-type electrode. The antenna's scapus was mounted on a negative electrode, and the flagellum was positioned on the positive electrode for signal acquisition.

Every contact point between the antenna and the electrode was coated by applying an electrode gel (Spectra® 360, Parker Laboratories Inc., Fairfield, NJ, USA). An electroantennographic detector (EAD) was connected to the electrode to detect the electric activity of sensory neurons of the antenna, and these signals were amplified 500 times (AC/DC UN-6; Syntech, Hilversum, Netherlands). A puff system was connected to the electroantennographic detector, responsible for controlling the flow intensity of the chemical stimulus puffed on the antenna. This system was connected to a Pasteur's glass pipet (to insert the chemical stimulus) and a glass cylinder (to insert the antenna preparation).

Treatment was represented by a synthetic hexanal solution (the major-produced volatile compound of cut culms of bamboo; for details, see Results) prepared in isopropanol solvent. Control was represented by isopropanol. An aliquot of 10 µL of the treatment solution (containing 1000 µg of hexanal) was applied onto a filter paper-strip (50 mm long × 5 mm width). After 1 min of the application, the paper-strip was inserted into a Pasteur's glass pipet. Activated-charcoal-filtered air was puffed directly over the antenna preparation, which was exposed to the chemical stimulus. After each puff, a delay time of 1 min was adopted for the antenna recovering.

Each tested antenna (representing a single adult *D. minutus*) for both treatment and control was considered a replicate. The results obtained for each replicate's treatment and control were represented by the mean value of EAG responses resulting from two puffs. For statistical analysis, mean EAG responses were normalized by the division of the mean of the treatment (i.e., hexanal) by the mean of control in each replicate. A total of 20 beetles were tested (10 males: 10 females). EAG signals were recorded and analyzed by Syntech GcEAD software (version 4.6) (Syntech, Hilversum, Netherlands).

3.2.6. Field Bioassay of Synthetic Volatile Organic Compounds Produced by Cut Culms of Bamboo

Synthetic volatile organic compounds of bamboo, the candidate to attractants for *D. minutus*, were evaluated in the field in a ~0.8 ha area of common bamboo, *B. vulgaris* in Piracicaba, SP, Brazil (22°44'51" S, 47°34'55" W; 591 m altitude). Custom-made cross-vane traps were installed in the field (translucent polyethylene terephthalate glycol panels; 24 cm long × 19.5 cm width). Translucent traps appear to be neutral concerning physical factors affecting the insects' response to semiochemicals (*e.g.*,

colors and silhouettes) [11,12]. Collection jars (500 ml) were attached to the trap funnels and filled with 250 ml aqueous solution of propylene glycol (20%) to instantly kill, prevent escape, and conservation of the captured insects.

Traps were suspended from inverted L-shaped hungers of PVC pipe (1.8 m height × 2.5 cm i.d.). Trap interception panels have been hung at ~1.2 m from the ground level. Lures consisted of translucent polyethylene press-seal sachets (5 cm × 7 cm, 80 μm wall thickness, Daiso Ind. Co., Hiroshima, Japan) containing a dental cotton roll (Roeko Luna no. 1, Coltène/Whaledent Co., Langenau, Germany) loaded with 1-mL solution of synthetic volatiles in isopropanol. Control was represented by 1 mL of neat isopropanol, which was selected as a negative control because it has been used as a standard solvent for pheromones of wood-boring beetles [13] and appears not influenced the response of beetles at the dose adopted in this study. Lures were hung in the central open slot of traps. Lures were replaced every three days to prevent the complete depletion of the minor compounds. Treatments were assigned randomly to traps in blocks, and each block contained one trap of each treatment. Traps were placed 20 m apart, and blocks were spaced 20 m from each other. Traps were serviced daily for captured insects, at which time treatments were changed one position within blocks to control positional bias [12].

We adopted the following criteria to select the volatile candidate compounds that would be tested in the field assays: (i) the six most abundant compounds emitted by the cut culms of bamboo and with the frequency of detection of 100% in the headspace extracts at the peak of beetles' attacks; (ii) compounds that elicitated significant EAG responses on adult *D. minutus*. The dose of each compound per lure was based on its relative proportions to the major-produced compound (i.e., hexanal) determined from quantitative analysis of headspace extracts (for details, see Results, Table 1). Definition of the standard dose of hexanal in the lures was based on successful preliminary field tests (data not shown).

Dinoderus minutus is a multivoltine species with heavily overlapping generations throughout the year. Populations of *D. minutus* are frequently active in the field under tropical climate [4]. Therefore, the period for conducting field bioassays was defined at random. Field bioassays were conducted from 3 December 2020 to 4 January 2021.

Captured insect specimens were stored in 50-mL centrifuge tubes (NEST Biotechnology Co., Wuxi, Jiangsu, China) filled with 70% ethanol until identification. In the laboratory, *D. minutus* adults were identified based on morphological characters [14,15]. Because adults of this species do not have conspicuous sexual dimorphism indicated from external morphological characters [16], sex determination was based on the extrusion of the genitalia of every single specimen.

Collection of beetles were made under ICMBio permit #61549-3, issued by the Brazilian Ministry of the Environment. Voucher specimens of *D. minutus* have been deposited in the "Luiz de Queiroz" Museum of Entomology, Department of Entomology and Acarology (USP/ESALQ), Piracicaba, SP, Brazil, under register codes ESALQENT000063 to ESALQENT000072.

3.2.6.1. First Field Trial – Screening for Attractant Blends

This experiment comprised the following treatments: i) Hexanal 200 mg; ii) Blend 4 (hexanal 200 mg + octanal 5 mg + nonanal 10 mg + decanal 10 mg); iii) Blend 5 (hexanal 200 mg + heptanal 10 mg + octanal 5 mg + nonanal 10 mg + decanal 10 mg); iv) Blend 6 (hexanal 200 mg + heptanal 10 mg + octanal 5 mg + nonanal 10 mg + decanal 10 mg + 2-pentylfuran 30 mg); v) Control (1 ml of neat isopropanol). Four replicates (blocks) were adopted for each treatment.

3.2.6.2. Second Field Trial – Subtractive Evaluation of the Attractant Blends

This experiment was based on the results of the "first field trial" and included the following treatments: i) Blend 4 (hexanal 200 mg + octanal 5 mg + nonanal 10 mg + decanal 10 mg); ii) Blend 4 – Decanal; iii) Blend 4 – Nonanal; iv) Blend 4 – Octanal; v) Blend 4 – Hexanal; vi) Control (1 ml of neat isopropanol). Four replicates (blocks) were adopted for each treatment.

3.2.6.3. Third Field Trial – Defining the Bonafide Attractant

This experiment was based on the results of the "second field trial" and included the following treatments: i) Hexanal + nonanal + decanal; ii) Hexanal + nonanal; iii) Hexanal + decanal; iv) Nonanal + decanal; v) Control. The dose of each compound in the lures was the same as previously mentioned. Four replicates (blocks) were adopted for each treatment.

3.2.7. Statistical Analysis

Mean EAG responses of adult males and females of *D. minutus* between treatment and control were compared by ANOVA followed by Tukey's post hoc test at 1% probability.

The mean number of newly-built gallery holes by adult *D. minutus* on cut culms of bamboo at different periods after cutting the plants were compared by ANOVA followed by Tukey's post hoc test at 5% probability.

Differences between treatment mean in the number of beetles caught in each field experiment were tested individually for species (represented by at least ten specimens), using the nonparametric Friedman's test (PROC FREQ, option cmH) [17] because the assumptions of ANOVA were violated by heteroscedasticity [18]. Replicates were considered as a block and collection date, and replicates with zero captures were excluded from the analysis. In recognition of the multiple statistical tests of treatment effects, the significance level was adjusted by the number of species included in the analyses according to the Bonferroni procedure [19]. Pairs of means were compared using the Ryan-Einot-Gabriel-Welsch Q multiple range tests, which control the Type I experiment-wise error rate [17]. The sex ratio of adult *D. minutus* caught with traps baited with the bonafide attractant was compared to a nominal ratio of 0.5 with 95% Clopper–Pearson exact confidence intervals at 5% probability [20].

3.3. Results

3.3.1. Attack Intensity of Adult D. minutus to Cut Culms of Bamboo Over Time

Bamboo culms began to be attacked by *D. minutus* adults within 24 h after cutting (mean ± SE of 0.55 ± 0.21 beetle gallery holes). Thereafter, the attack intensity becomes progressively higher over time, reaching 9.36 ± 1.22 beetle gallery holes 35 days after cutting. At this point, the attack intensity was significantly higher ($Q_{5,60}$ = 4.1630, $p < 0.05$) compared to 7, 14 and 21 days (Figure 1).

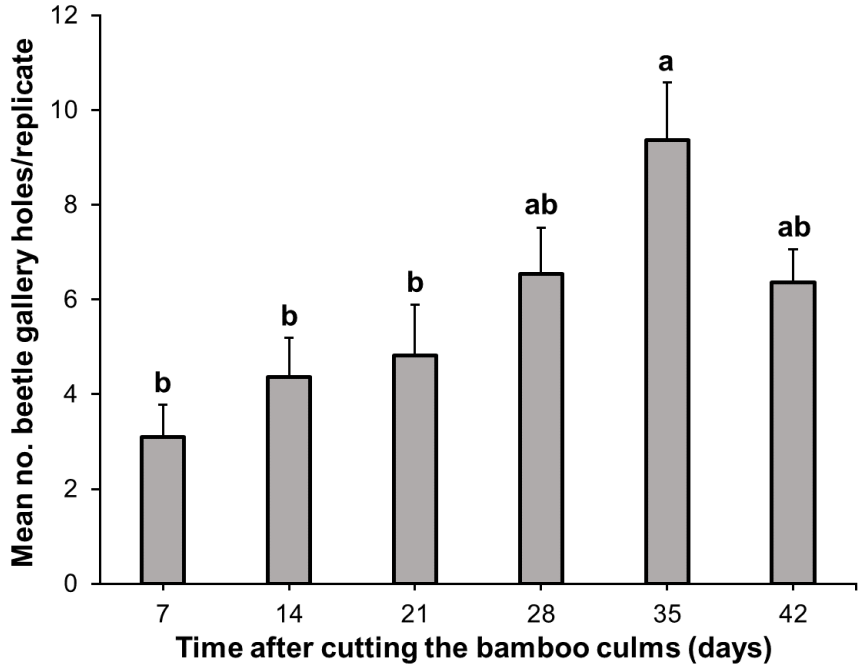

Figure 1. Mean (± SE) number of newly-built gallery holes by adult beetles of *Dinoderus minutus* on cut culms of bamboo over time. Means followed by the same letters are not significantly different according to the Tukey test at 5% probability.

3.3.2. Volatile Organic Compounds Produced by Cut Culms of Bamboo

A total of 24 volatile organic compounds were identified from the headspace extracts of cut culms of bamboo 35 days after cutting the plants (Figure 2; Table 1). Volatile compounds detected after 16 min (retention time) represented only contaminants of the aeration system (also detected in the control extracts) or residual artifacts from the GC column [21] and were excluded from the analysis.

Aldehydes represented the predominant functional group of volatile compounds produced by bamboo culms (86.5% of the total amount). The most abundant compounds were C_6-C_{10} aliphatic saturated aldehydes and 2-pentylfuran, accounting for 92.3% of the total amount of volatile compounds and frequency of 100% in the headspace extracts. The major-produced compound was hexanal (70.9% of the total) followed by 2-pentylfuran (8.8%). The released amounts of the C_7-C_{10} aliphatic saturated aldehydes were similar (Figure 2; Table 1).

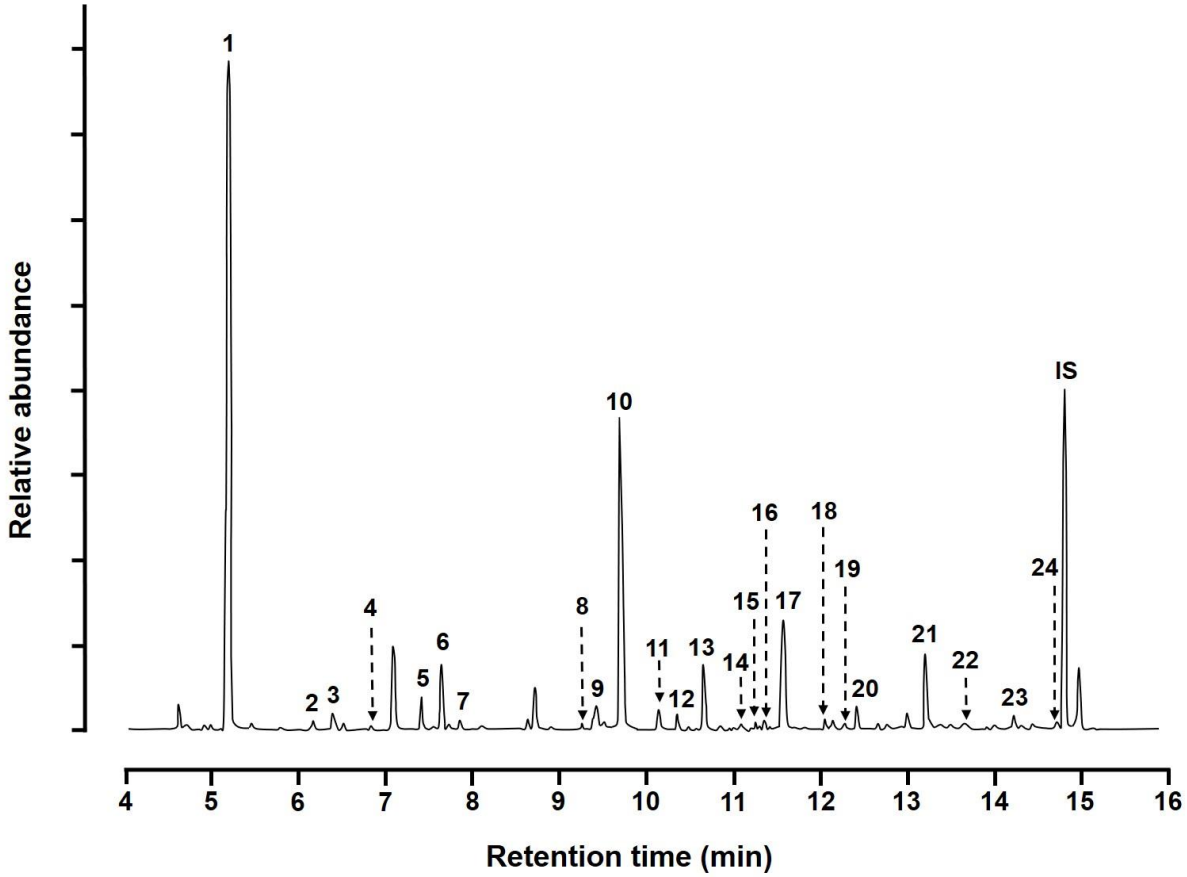

Figure 2. Representative total ion chromatograms of headspace volatile extracts from cut culms of bamboo plants at the peak of the attack by adult *Dinoderus minutus*. Numbers on the peaks represent the volatile organic compounds produced by bamboo culms, which are represented in Table 1. IS = internal standard (nonyl acetate). Unidentified peaks represent contaminants of the aeration system or residual artifacts from the GC column.

Table 1. Volatile organic compounds produced by the bamboo culms (*Bambusa vulgaris*) at the peak of attack intensity by adult *Dinoderus minutus* (35 days after cutting the plants).

Peak number	Compound	RI[1] on Rtx-1 capillary column	Released amount (ηg/100 g of bamboo/24 h)	Relative amount [%]
1	Hexanal*	770	5983.1±435.2	70.85
2	2-Ethyl-3-methyl-1-butanal	801	32.7±1.8	0.39
3	(*E*)-2-Hexenal	830	76.0±4.2	0.90
4	(*Z*)-2-Hexenol	835	2.8±1.2	0.03
5	2-Heptanone	872	99.0±5.6	1.17
6	Heptanal*	884	270.0±10.9	3.20
7	Pentyloxirane	900	40.7±6.9	0.48
8	1-Octen-3-one	959	11.1±0.9	0.13
9	Octanal*	980	149.1±12.3	1.77
10	2-Pentylfuran*	989	739.8±36.3	8.76
11	Ethyl hexanoate	998	10.4±0.7	0.12
12	3-Ethyl-2-methyl-1,3-hexadiene	1029	1.5±0.5	0.02
13	2-Ethyl-1-hexanol	1040	63.3±7.1	0.75
14	1-Octanol	1064	16.0±1.5	0.19
15	2,5-Dimethylcyclohexanol	1070	35.4±5.8	0.42
16	3,5-Dimethylcyclohexanol	1076	21.4±1.7	0.25
17	Nonanal*	1082	321.6±34.0	3.81
18	5,9-Dimethyl-2-decanone	1093	21.7±2.0	0.26
19	2-Isopropyl-5-methyl-2-hexenal	1106	15.3±1.5	0.18
20	(*E*)-2-Nonenal	1136	80.7±15.5	0.96
21	Decanal*	1188	335.0±73.6	3.97
22	Undecanal	1292	39.8±2.6	0.47
23	(*Z*)-6,10-Dimethyl-5,9-undecadien-2-one (=Geranyl acetone)	1434	17.7±1.0	0.21
24	1-Dodecanol	1473	47.1±7.5	0.56

[1]Kovats retention index. [2]Mean amounts (± SE) of volatile organic compounds released by the bamboo culms. *Compound tentatively identified by co-injection with authentic standards.

3.3.3. Electroantennography of the Bamboo Borer D. minutus

The synthetic hexanal at the dose of 1000 µg elicited significant EAG responses on *D. minutus* males (1.95 ± 0.22 mV) ($Q_{2,18}$ = 4.0707, p < 0.0005; Figure 3A) and females (1.51 ± 0.09 mV) ($Q_{2,18}$ = 4.0707, p < 0.0001; Figure 3B).

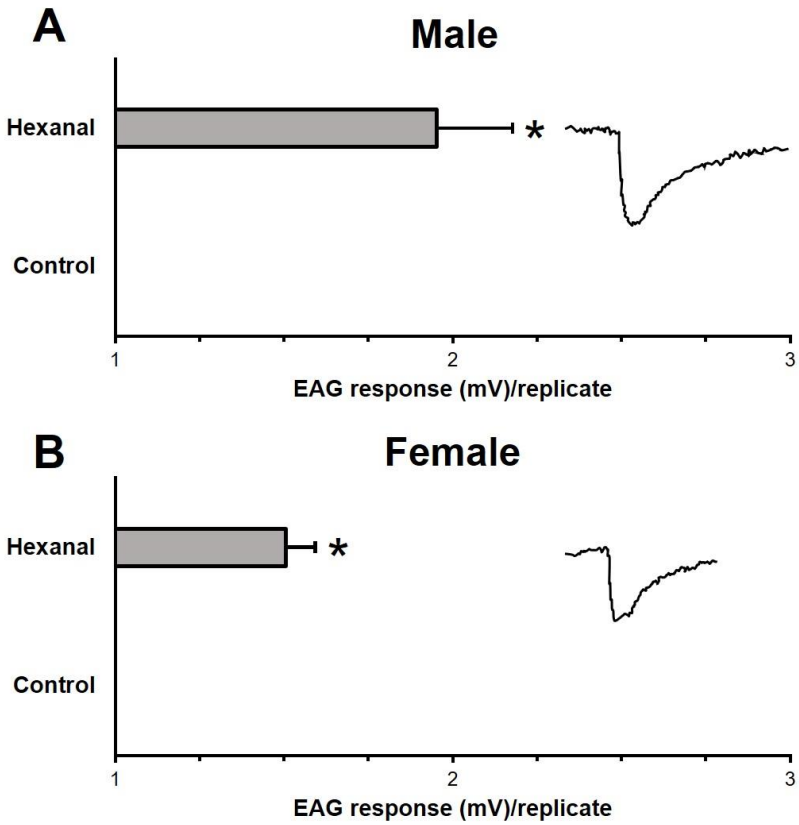

Figure 3. Mean (± SE) electroantennographic (EAG) responses of adult males (**A**) and females (**B**) of *Dinoderus minutus* to synthetic hexanal. Means followed by (*) within a panel are significantly different according to the Tukey test at 1% probability. Control = neat isopropanol. EAG responses are expressed in millivolts (mV).

3.3.4. Field Bioassay of Synthetic Volatile Compounds produced by Cut Culms of Bamboo

The first field trial shows that *D. minutus* adults were equally attracted to all tested blends of volatile compounds compared to control. The presence of heptanal and 2-pentylfuran in the blends is unnecessary to trigger the beetles' attraction. Also, hexanal alone do not attract the adult beetles (Figure 4).

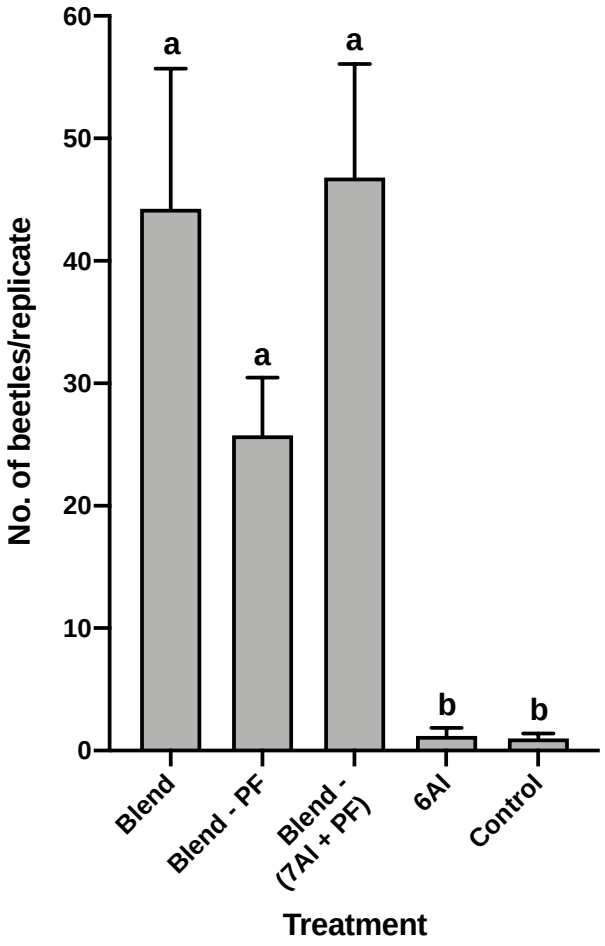

Figure 4. Mean (± SE) number of adults of *Dinoderus minutus* caught with traps containing synthetic volatile compounds produced by cut culms of bamboo plants. Treatment abbreviations: Blend = hexanal + heptanal + octanal + nonanal + decanal + 2-pentylfuran; PF = 2-pentylfuran; 6Al = hexanal; 7 Al = heptanal. Control = neat isopropanol. Type of attractant lure had a significant effect on trap catch of adult beetles ($Q_{4,75} = 53.2$, $p < 0.0001$). Means followed by the same letter are not significantly different according to the Ryan-Einot-Gabriel-Welsch Q multiple range test at 5% probability.

The second field trial demonstrates that the ternary blends, composed of hexanal + nonanal + decanal and hexanal + octanal + decanal, attracted similar numbers of *D. minutus* adults. This bioassay revealed a critical necessity of the presence of both hexanal and decanal in the blends (Figure 5).

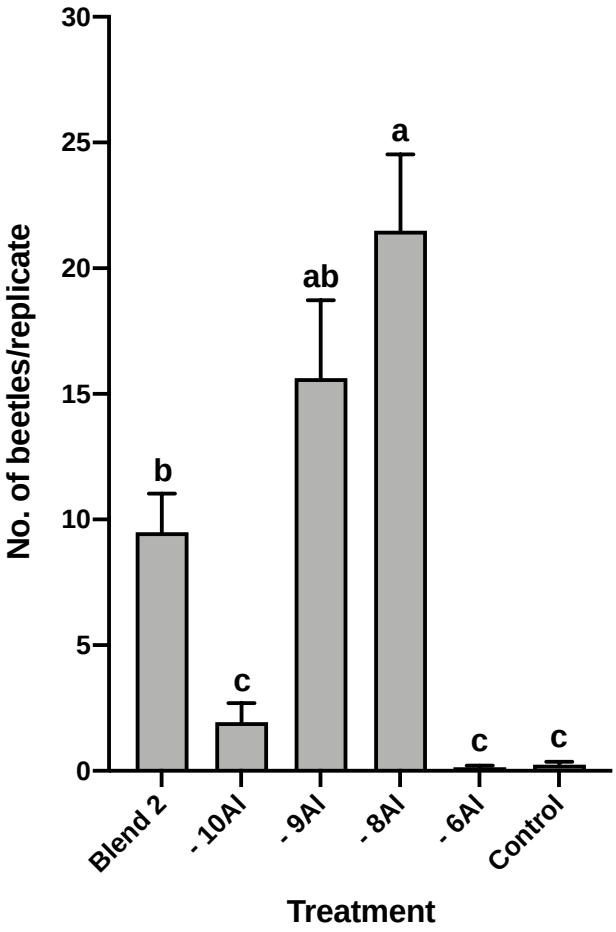

Figure 5. Mean (± SE) number of adults of *Dinoderus minutus* caught with traps containing synthetic volatile compounds produced by cut culms of bamboo plants. Treatment abbreviations: Blend 2 = hexanal + octanal + nonanal + decanal; - 6 Al = Blend 2 - hexanal; - 8 Al = Blend 2 - octanal; - 9 Al = Blend 2 - nonanal; - 10 Al = Blend 2 - decanal; Control = neat isopropanol. Type of attractant lure had a significant effect on trap catch of adult beetles ($Q_{5,96}$ = 73.3, p < 0.0001). Means followed by the same letter are not significantly different according to the Ryan-Einot-Gabriel-Welsch Q multiple range test at 5% probability.

The third field trial validated the blend composed of hexanal + decanal as the bonafide attractant for *D. minutus* adult (Figure 6), suggesting a synergistic interaction between these aldehydes. Overall, 149 adults of *D. minutus* (80 males and 69 females) were caught in traps baited with hexanal + decanal. The sex ratio of beetles attracted to traps with hexanal + decanal did not differ from the expected ratio of 0.5 (46.31% females, 95% Clopper-Pearson Exact Confidence Intervals: 0.3811-0.5465, p = 0.3675).

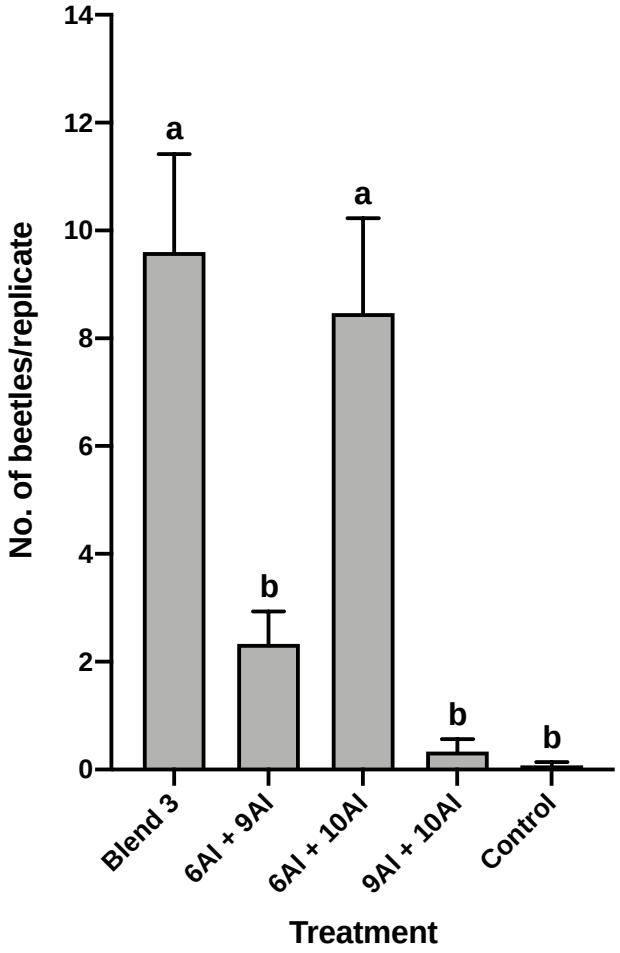

Figure 6. Mean (± SE) number of adults of *Dinoderus minutus* caught with traps containing synthetic volatile compounds produced by cut culms of bamboo plants. Treatment abbreviations: Blend 3 = hexanal + nonanal + decanal; 6 Al = hexanal; 9 Al = nonanal; 10 Al = decanal; Control = neat isopropanol. Type of attractant lure had a significant effect on trap catch of adult beetles ($Q_{4,75} = 48.3$, $p < 0.0001$). Means followed by the same letter are not significantly different according to the Ryan-Einot-Gabriel-Welsch Q multiple range test at 5% probability.

3.4. Discussion

Adults of *D. minutus* were attracted to newly-cut culms of the bamboo, *B. vulgaris*, and started to build gallery holes within 24 h after cutting the plants. The same results were obtained by Garcia and Morrell [6], which observed that *D. minutus* adults started to build galleries within 24 h after cutting the *B. vulgaris* culms. The attack intensity on cut culms of bamboo becomes progressively higher over time. This phenomenon appears to be linked with the decrease of culm moisture and secondary phenolic compounds in the tissues of the bamboo culms [10,22].

The functional group of aldehydes represents almost 86% of the total volatile emissions of cut culms of *B. vulgaris* at the peak of beetles' attack. Similary, the relative content of aldehydes was the highest among the volatile emissions of bamboo shoots of *Bambusa emeiensis* L.C.Chia & H.L.Fung (=*Neosinocalamus affinis*) and *Pleioblastus amarus* (Keng) Keng f. (Poaceae) [23]. Aldehydes and alcohols were the most abundant volatile compounds of oil obtained from culms of the bamboo *Phyllostachys edulis* (Carrière) J.Houz. (=*Phyllostachys pubescens*) (Poaceae) [24]. Furthermore,

aldehydes and alcohols represented the main volatile compounds of the cut black bamboo *Phyllostachys nigra* (Lodd. ex Lindl.) Munro, which are more abundant compared to intact plants emissions [25]. The profile of *B. vulgaris* culms' volatile organic compounds is similar to other bamboo species [23–25]. The biologically active aldehyde attractants for *D. minutus* (i.e., hexanal + decanal) were also identified among the volatile emissions of the bamboo *P. edulis* [23]. It is reasonable that *D. minutus* locate suitable host plants of bamboo belonging to these different taxa using the same binary blend consisting of hexanal + decanal that these plants may produce in common. Investigation of the profile of volatile compounds emitted by different bamboo species and its correlation with the attraction or repellence of *D. minutus* deserves further studies.

Adults of *D. minutus* were attracted to different blends of volatile organic compounds emitted by cut culms of *B vulgaris* in the field. However, a blend of two saturated aliphatic aldehydes (i.e., hexanal + decanal) was verified as the bonafide attractant for adult males and females of *D. minutus*, once the presence of other compounds in the blends not enhanced the attraction. This blend is a long-range chemical cue that can supposedly provide essential information for dispersing adults of *D. minutus* regarding the location of suitable bamboo culms for feeding and reproduction.

Volatile aldehydes are known as secondary products of lipid oxidation [26–29]. Hexanal represented the major-produced volatile compound by cut culms of bamboo. This compound is considered the most important indicator of lipid oxidation in food products, resulting from linoleic acid's autoxidation [26,29–31]. We hypothesize that hexanal and decanal as well as the other aliphatic aldehydes are products of oxidation of the starch-associated lipids present in the parenchyma of culms of decaying and dead bamboo plants. Furthermore, such aldehydes can potentially provide essential information regarding the nutritional suitability of the food resource for *D. minutus* adults.

The initial preference of *D. minutus* for the bamboo *B. vulgaris* [5] may be due to the emissions of the volatile compounds hexanal and decanal. In a close-range scenario, the nutritional properties, secondary chemistry [10,32,33], and moisture content [22] of bamboo plants' culms consist of important factors in the host-plant selection by *D. minutus* adults. For instance, females of *Dinoderus ocellaris* Stephens (Bostrichidae) appear to chew the tissues of bamboo culms to determine the nutritional suitability for feeding before boring galleries [34].

There is little consistent evidence of the primary attraction of species belonging to the family Bostrichidae to plant-derived kairomones, for instance, the lesser grain borer, *Rhyzopertha dominica* (Fabricius) [7], and the West African ghoon beetle, *Dinoderus bifoveolatus* (Wollaston) [9]. The attraction of *R. dominica* was enhanced by the combination of male-produced aggregation pheromone with ethanol [35], which is produced by grains of cereal host plants after harvesting and under storage conditions [36].

Adults of weevils (Coleoptera: Curculionidae) and flat grain beetles (Coleoptera: Silvanidae) that are known as noxious pests of stored-food products appear to use volatile aldehydes as plant-derived kairomones, which are well-known volatile products of lipid oxidation of cereal grains [27,31,36]. Adult males and females of the maize weevil, *Sitophilus zeamais* (Motschulsky) (Coleoptera:

Curculionidae) were attracted to a blend of volatile compounds which consist of hexanal + (*E*)-2-heptanal + octanal emitted from grains of *Zea mays* (L.) (Poaceae). However, individually, these aldehydes are not attractive to these beetles, suggesting a synergistic interaction [37]. The attraction of two other important cereal pests, the granary weevil, *Sitophilus granarius* (L.), and the rice weevil, *Sitophilius oryzae* (L.) were also observed for volatile aldehyde compounds originated from cereal grains [38,39]. The sawtoothed grain beetle, *Oryzaephilus surinamensis* (L.) (Coleoptera: Silvanidae) was attracted to various aliphatic aldehydes emitted by food products, including hexanal, octanal, and nonanal [40,41]. The merchant grain beetle, *Oryzaephilus mercator* (Fauvel), and *O. surinamensis* were attracted to a range of saturated aliphatic aldehydes originating from food products, including hexanal and decanal. Additionally, the combination of a blend composed of hexanal + octanal + nonanal with the aggregation-pheromone components of these cucujoid beetles substantially enhanced its attraction [42].

The saturated aliphatic aldehydes that we found to comprise a bonafide attractant blend for *D. minutus* (i.e., hexanal + decanal) possess relatively low cost and are readily-available in chemical manufacturers. Attractant lures based on these inexpensive compounds could be incorporated in trapping systems for detection and monitoring the populations of *D. minutus* wherever it occurs. In some conditions of raw timber of bamboo storage, traps baited with this attractant could be applied for early detection of pest infestations, mass trapping, and attraction of adult beetles to insecticide-treated areas [43,44]. Besides, these aldehydes can be exploited as kairomones for other insect pests that breed on harvested bamboo culms and stored cereal grains [40], once these compounds are highly conserved among lipid-rich monocot plants. Nevertheless, further studies are necessary to determine the aldehydes' optimal release ratios for *D. minutus* attraction.

3.5. Conclusions

Adults of *Dinoderus minutus* were attracted to newly-cut culms of the bamboo, *Bambusa vulgaris*, and started to build gallery holes within 24 h after cutting the plants. The attack intensity on cut culms of bamboo by the adults increased over time. At the peak of the beetles' attacks, the cut culms of bamboo released substantial amounts of some volatile organic compounds. In the field, synthetic blends of these bamboo-produced volatile compounds attracted *D. minutus* adults. The bonafide blend of compounds attracted both adult males and females of *D. minutus*. This blend was identified as the chemical cue involved with the host-plant location by the adults of *D. minutus*. Traps baited with this semiochemical-based attractant have the potential to be exploited in the monitoring and control of *D. minutus*.

Author Contributions

H.L.R., M.S., W.D.S. and J.M.S.B. conceived the study. H.L.R. obtained the volatile extracts and identified the semiochemical candidates. F.G.G. conducted the electrophysiological tests. H.L.R. and F.G.G. carried out the field bioassays. H.L.R. identified the collected specimens. W.D.S. and H.L.R. performed the statistical analyses. J.M.S.B. obtained the funding. H.L.R. wrote the manuscript draft. W.D.S., J.M.S.B. and M.S. revised and edited the manuscript draft. All authors have read, reviewed and contributed to the final version of the manuscript.

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Conflicts of Interest

The authors declare no conflicts of interest.

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