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Mate Recognition by the Green Mate Borer, *Hedypathes betulinus* (Coleoptera: Cerambycidae): the Role of Cuticular Compounds

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Abstract Green mate, *Ilex paraguariensis* (Aquifoliaceae), is native to South America and its range includes the south of Brazil. *Hedypathes betulinus* (Coleoptera: Cerambycidae) is considered the most important insect pest of green mate, causing severe damage to plants and significant economic losses. In previous work, laboratory assays were performed to document the mating sequence of this species and the male long-range sex pheromone was identified. Previous reports of the mating sequence suggested that males recognize females only after antennal contact and consequently the existence of a contact sex pheromone. To test the hypothesis that mate-recognition in *H. betulinus* is mediated by a contact sex pheromone, this study analyzed male and female body extracts by GC-MS. Extracts were fractionated to separate the compounds by polarity, producing hexane, cyclohexene and ether fractions. Active fractions were identified with laboratory bioassays. Bioassays were performed by introducing female cadavers to live males and male copulation response scored (±). In assays using solvent washed female cadavers, males did not recognize female cadavers, but attempted copulation with female cadavers with the extract re-applied. Cumulatively, these results suggest that a contact sex pheromone on the cuticle of females mediates mate recognition. Laboratory bioassays and analysis of the female solvent extracts produced by fractionation, individually and in binary and ternary combinations, suggests that the more polar compounds present in the ether fraction are crucial to mate recognition in this species.

Keywords Longhorn beetle · mate recognition · contact pheromone · cuticular compounds

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Introduction

Green mate, *Ilex paraguariensis* (Aquifoliaceae), is native to South America and its distribution includes Brazil, Argentina and Paraguay (EMBRAPA 2010). In 2016 the South of Brazil produced 346,769 tons of leaves of green mate, resulting in an income of approximately US$129,503,571.00 (IBGE 2016). The state of Paraná is the biggest producer of green mate in Brazil (86.4% of the national production), followed by Santa Catarina (8.3%)

and Rio Grande do Sul (5.2%) (IBGE 2016). *Ilex paraguariensis* is cultivated in approximately 180,000 rural properties distributed in 480 municipalities of Southern Brazil (EMBRAPA 2010). Its harvest has an important economic and social impact in the region, being the main source of income for small producers and generating ca. 700,000 jobs, especially during harvest season (from June to August) (EMBRAPA 2010). *Ilex paraguariensis* is the economic base of many Southern municipalities of Brazil and the control of insect pests is one of the biggest challenges green mate producers must overcome (Fonseca and Zarbin 2009).

Hedypathes betulinus (Coleoptera: Cerambycidae: Lamiinae) is the most important insect pest of green mate, resulting in severe damage to plants and significant economic losses (Cassanello 1993; Guedes et al. 2000). Adult beetles are approximately 25 mm in length and are easily identified by their color pattern, black color with white bristles distributed mainly on the elytra, and the long antennae, characteristic of many Cerambycidae (Iede and Machado 1989; Cassanello 1993). Female *H. betulinus* oviposit one egg per branch, usually in openings in the trunk or in the base of the stalk of plants, but they can also oviposit in exposed roots or recently clipped parts of the plant (Cassanello 1993; Penteado 1995). The larvae feed in the branches and twigs, building longitudinal subcortical galleries that block natural sap flow of the plant (Cassanello 1993).

Fonseca and Zarbin (2009) used laboratory assays to document the mating sequence of *H. betulinus*. The mating sequence of *H. betulinus* was described as (i) the female walks toward the male suggesting that chemical and/or visual signals produced by the male attracts the female; (ii) the female touches the male antennae with her antennae; (iii) immediately after male-female antennal contact is made the male mounts the female, rotates 180° and grasps the female pronotum or elytra with his forelegs and (iv) the male attempts to copulate with the female bending his abdomen towards the female and coupling male-female genitalia. Fonseca and Zarbin (2009) observed that males only tried to copulate with females after antennal contact was made, suggesting the presence of contact pheromones in *H. betulinus*. After mating, mate-guarding occurs (i.e. the male grasps the female pronotum or elytra and remains mounted on the female after copulation), preventing copulation with other males and ensuring paternity (Fonseca and Zarbin 2009). Mate location in Cerambycidae generally involves pheromones that act over long and short distances (Allison et al. 2004; Hanks and Millar 2016). Volatile pheromones active over long-distances can be categorized as sex pheromones, which are usually produced by females and attract only males, and aggregation-sex pheromones, generally produced by males and attract both sexes (Cardé 2014). The sex pheromone of *H. betulinus* was identified as a mixture of (*E*)-geranylacetone, (*R*/*S*)-(*E*)-fuscumol and (*R*)-(*E*)-fuscumol acetate (Fonseca et al. 2010; Vidal et al. 2010). This pheromone is produced by males and the ternary mixture of those compounds (alone and in combination with host plant volatiles) attracted females in laboratory assays (Fonseca et al. 2010). Contact pheromones are also important in the mating recognition system of Cerambycidae. Evidence exists for several species that males recognize females through contact chemoreception using the antennae (Hanks 1999; Allison et al. 2004; Ginzel et al. 2006).

The primary objective of this study was to test the hypothesis that male and female cuticular profiles differ and mediate mate recognition in *H. betulinus*. Supporting secondary objectives were the identification of the fractions necessary and sufficient for mate recognition and the identification of candidate contact sex pheromone components.

Material and Methods

Insects

Adult *H. betulinus* of unknown age were obtained from green mate crops of the Vier, Indústria e Comércio do Mate Ltda, in São Mateus do Sul - PR, Brazil (25°54′46”S 50°33′46”W) from September, 2016 – April, 2017 and from November, 2017 – March, 2018. All insects were transferred to the Laboratory of Semiochemicals, Chemistry Department of the Federal University of Paraná (UFPR) where they were separated by sex [the scape of the antennae and the femur of all three pairs of legs are larger in males than in females (Cassanello 1993)], and placed individually in round capped plastic containers Plaszom® (9.5 × 5.5 cm) with holes on the side and cap to allow air circulation. Fresh green mate twigs (*I. paraguariensis*) of approximately 8 cm length without the leaves were provided to all beetles twice a week. All insects were maintained under controlled conditions at 25 ± 3 °C and 50 ± 10% RH under a 12:12 L:D cycle for at least 10 days prior to assay.

Mating Bioassays

Ten female *H. betulinus* were kept individually under controlled conditions (described above) with free access to food for at least 10 days before being sequentially manipulated to generate the following treatments (i.e., all females were used sequentially in treatments 1, 2 and 3):

1. Females were killed by freezing at −20 °C for one hour, warmed to room temperature for 15 min, and presented individually to a male in order to confirm that the chemical mate recognition signals were intact (i.e., males recognize and attempt copulation with dead females).
2. Female cadavers were immersed individually in 5 mL of double distilled hexane for 10 min to remove the chemical mate recognition signals. The first rinse of each female was concentrated under gentle air flow to a final volume of 0.5 mL and stored at −20 °C. Females were then extracted a second time by placing them in a 200 mL Pyrex® Sohxlet, at approximately 68 °C, containing 400 mL of hexane for 12 to 24 h. Each extracted female cadaver was air dried overnight, to allow the complete evaporation of the solvent, and individually introduced to a male to determine if mate recognition occurred (i.e., if mate recognition is mediated by compounds present on the female cuticle, males should not recognize solvent extracted female cadavers).
3. One female equivalent (FE) was reapplied drop wise to the elytra of the same female cadaver it was extracted from (= reconstituted females) and subsequently presented to a male (i.e., if mate recognition is mediated by compounds present on the female cuticle, males should recognize solvent-extracted female cadavers with the cuticular profile reconstituted on the elytra).

Treatments had an interval of one to three days between them. Each female was sequentially presented to two individual males in all treatments.

Bioassays were conducted by placing a male *H. betulinus* in a Petrii dish lined with filter paper (Qualy® 9 cm diameter). After five minutes a female *H. betulinus* cadaver was presented to a conspecific male (e.g., an untreated cadaver, an extracted cadaver or a reconstituted cadaver). Using tweezers, the female cadaver was slowly moved towards the conspecific male until contact was made between male and female antennae, simulating the beginning of the mating sequence of this species (in this species the mating sequence begins when the female approaches the male and touches the male antennae with her antennae) (Fonseca and Zarbin 2009). Each female was manipulated in order to produce the three different treatments (killed and cuticular profile intact, cuticular profile extracted and cuticular profile re-applied) and an individual male was sequentially assayed with the same female in each treatment state.

Thirteen females and 25 males were used in the mating bioassay (the experimental design called for 26 males, but one male died during the bioassay). Observations were terminated after the female cadaver touched the male antennae with her antennae three times with no response from the male or once males were observed to make antennal contact with the female (behavior 1), mount (behavior 2), rotate 180° on top of the female (behavior 3) and attempt copulation (behavior 4) (the bioassay was always terminated before copulation occurred). Whenever a male attempted to copulate with a female cadaver that had been solvent extracted, the female cadaver was rinsed one more time and introduced to the same male. If males still attempted copulation with female cadavers that were submitted to an additional 12 h of solvent extraction in the Soxhlet, these males and females were excluded from bioassays (3 female cadavers and the associated 6 males were excluded on the basis of this condition). Only rinsed female cadavers that did not induce copulation attempts were used in the reconstituted female treatment. All bioassays were conducted from 4 to 24 March, 2017 and from 29-March to 1-April, 2018 between 14:00 and 18:00 h. The response of males to females was scored as 1 when the behavior was observed, or 0 when the behavior was not observed.

Extraction and Fractionation of Cuticular Compounds

In January, 2017, 30 female *H. betulinus* were killed by freezing at −20 °C for one hour and then immersed individually in 5 mL of double distilled hexane for 10 min ($n = 30$). Female extracts were combined and stored in a 250 mL glass media bottle (Duran®) at −20 °C. The extracts were then transferred to a 250 mL round bottom flask and the Duran® glass media bottle (i.e., in which the female extracts were previously stored) was rinsed with 3 aliquots of 2 mL of double distilled

hexane and added to the round bottom flask. The 250 mL round bottom flask was placed onto a Buchii Rotavapor® with water bath set at 30 °C under gentle vacuum and 110 rpm until the organic extract was reduced to a volume of 2.1 mL and then transferred to a 15 mL disposable vial. The 250 mL round bottom flask was rinsed with 3 aliquots of 2 mL of double distilled hexane which was transferred to the 15 mL disposable vial containing the organic extract. The 15 mL disposable vial was capped with a Teflon septa and stored at −20 °C.

The female *H. betulinus* body wash extract was fractionated using silica gel impregnated with 10% $AgNO_3$. The silica was impregnated with 10% $AgNO_3$ according to the methodology described by Li et al. (1995). A solution of 0.3 g of silver nitrate with a few drops of distilled water was mixed with 3 g of silica and ground for 5 min in a ceramic mortar. Then, the ceramic mortar was covered with aluminum foil and dried in an oven at 200 °C for 4 h. The silica gel impregnated with $AgNO_3$ was transferred to a capped 10 mL disposable vial wrapped in aluminum foil and stored protected from light. Each sample was loaded on a column with 1.5 g of silica wetted with hexane. The column was rinsed with 1 mL of double distilled hexane to incorporate all the sample on the silica and gradually eluted with solvents in order to get the following fractions:

1. 6 mL of hexane (saturated hydrocarbons)
2. 6 mL of cyclohexene (unsaturated hydrocarbons)
3. 6 mL of ether (more polar compounds)

The final volume of each fraction was collected in 30 mL disposable vials capped with Teflon® septa and stored at −20 °C. Each fraction was diluted in double distilled hexane and divided in 0.5 mL female equivalents (FEs). FEs were placed in 2 mL disposable glass vials capped with Teflon® septa and stored in a freezer at −20 °C. The fractionation of female extracts was repeated in January, 2018, but using extracts of 15 female *H. betulinus* to obtain more fractions to be used in the bioassays executed from January to April, 2018.

Fraction Bioassays

Bioassays of individual fractions and the binary and tertiary blends were conducted from 24-March to 25-May, 2017 and 23-January to 29-March, 2018. *Hedypathes betulinus* females were manipulated as described above to produce female cadavers with no traces of mate recognition signals on their cuticle (rinsed females). Then, one FE of an individual fraction(s) was applied to the elytra of an *H. betulinus* female cadaver previously solvent extracted as described above.

An individual *H. betulinus* male was placed in a Petrii dish lined with filter paper (Qualy® 9 cm diameter) and after five minutes, the female cadaver with the fraction(s) applied was introduced. In total, 121 *H. betulinus* males were presented to 72 female cadavers with individual fractions, binary blends or the tertiary blend of fractions applied to their elytra. Males that displayed the mating behavior sequence after making antennal contact with the female carcass that was previously submitted to solvent extraction in the Sohxlet for more than 24 h were excluded from the analyses and female cadavers were similarly excluded (13 female cadavers and 26 males were excluded on the basis of this criterion). Males that died between treatments were also excluded from the analysis (one male in the H + C Fractions bioassay). Whenever possible, beetles were used just once, but beetles that were used more than once per bioassay had an interval of at least 72 h between bioassays (23 males were used in more than one bioassay). Beetles were never used more than once per treatment.

Comparison of Male and Female Fractions

Fractionation of *H. betulinus* cuticular extracts of cohorts ($n = 5$) of five females (i.e., 25 females extracted total) and cohorts ($n = 5$) of five males each (i.e., 25 males extracted total) were done and each fraction was analyzed in GC-MS. Unidentified compounds that appeared to have different concentrations in male and female hexane, cyclohexene and ether fractions were quantified through co-injection with the synthetic standard tetradecane (Sigma-Aldrich®) at 50 ppm.

Fuscumol Acetate Determination and Quantification

The presence of 6,10-dimethyl-5,9-undecadien-2-yl acetate (fuscumol acetate) in *H. betulinus* male and female crude extracts was detected by GC-MS analyses and its identification was confirmed by comparison with the literature (Fonseca et al. 2010) and co-injection with synthetic standards. Quantification of fuscumol acetate present in the male and female ether fractions was done through co-injection of an internal standard (tetradecane) (Sigma-Aldrich®) at 50 ppm. The

geometrical configuration of the double bond C-C of fuscumol acetate present in an ether fraction containing extracts of 15 *H. betulinus* females was determined through co-injection with synthetic standards of (*E*)- and (*Z*)-fuscumol acetate (6:4) at 50 ppm. The determination of the stereochemistry of fuscumol acetate present in ether fractions of female *H. betulinus* was done through comparison with synthetic standards of (*R*)- and (*S*)-fuscumol acetate (1:1) at 100 ppm. An aliquot of 2 mL of an ether fraction containing extracts of 15 female *H. betulinus* was concentrated to 100 μL under gentle air-flow prior to analyses.

Synthetic Fuscumol Acetate Bioassays

Bioassays of a female equivalent (FE) of (*R/S*)-, (*R*)- and (*S*)-fuscumol acetate and a male equivalent (ME) of (*R*)-fuscumol acetate were performed from 19-March to 21-May, 2018. Samples of synthetic compounds were prepared with the concentration of fuscumol acetate present in the cuticular extracts of each sex. *Hedypathes betulinus* females were manipulated as described above in order to produce rinsed female cadavers. After solvent extraction, one FE of synthetic (*R/S*)-, (*R*)- or (*S*)-fuscumol acetate or one ME of synthetic (*R*)-fuscumol acetate was applied to the elytra of each of 42 previously extracted female cadavers. Treated female cadavers were then individually introduced to 81 *H. betulinus* males in Petrii dishes as described above (see "Fraction Bioassays"). Each treated female cadaver was introduced to two individual males (three males died between treatments and were excluded from the analyses: one male in the FE of (*R/S*)-fuscumol acetate bioassay; one male in the FE of (*R*)-fuscumol acetate bioassay; one male in the ME of (*R*)-fuscumol acetate bioassay). Male beetles were used only once per bioassay.

Analytical Procedures

All GC-MS analyses were performed on a Shimadzu® QP2010 Plus GC-MS operated in splitless mode. An Agilent® DB-5 (30 m × 0,25 mm × 0,25 μm) capillary column was used under the following analytical conditions: initial temperature of 100 °C for 1 min increasing 7 °C/min until 270 °C, which was held for 30 min. Synthetic standards of (*R*)- and (*S*)-fuscumol acetate and the concentrated aliquot of the ether fraction of 15 female *H. betulinus* were analyzed with a Shimadzu® 2010 Gas Chromatograph with flame ionization detection (FID) equipped with a capillary β-DEX 325 column (30 m × 0.25 mm × 0.25 μm) operated in splitless mode under the following analytical conditions: isothermal of 100 °C for 400 min.

Statistical Analyses

The Fisher Exact Test with an experiment-wise error rate of .05 was used to compare male *H. betulinus* copulation response to female cadavers with the individual, binary or tertiary blends of fractions applied to the elytra to male copulation response to freeze-killed female cadavers. The Fisher Exact Test was also used to contrast all bioassays in pairs.

Shapiro-Wilk Test of Normality followed by Wilcoxon Signed Rank Test or Two Sample T-Test were performed to confirm the differences of quantities (ppm) of unidentified compounds between male and female ether fractions. Differences of quantities (ppm) of fuscumol acetate observed between male and female ether fractions were also confirmed through Shapiro-Wilk Test of Normality followed by Wilcoxon Signed Rank Test.

The Fisher Exact Test with an experiment-wise error rate of .05 was used to compare male *H. betulinus* copulation response to female cadavers with FE of synthetic (*R/S*)-, (*R*)- or (*S*)-fuscumol acetate or ME of synthetic (*R*)-fuscumol acetate applied to the elytra to male copulation response to freeze-killed female cadavers that were not solvent extracted. The Fisher Exact Test was also used to compare all synthetic fuscumol acetate bioassays and ether fraction bioassay in pairs. All analyses were done using the software $R_{3.4.0}$ (The R Foundation for Statistical Computing 2017).

Results

Mating Bioassays

All *H. betulinus* males ($n = 19$) assayed were observed to touch the freeze-killed female cadavers with their antennae (behavior 1), mount (behavior 2), rotate 180° on the top of the cadaver (behavior 3) and subsequently attempt copulation (behavior 4). These female cadavers were then solvent rinsed and introduced to the same 19 males in mating arenas. All males touched the rinsed female cadavers with their antennae but none mounted or attempted copulation with them (i.e., no positive

response for the second, third and fourth mating behaviors were observed).

Mate recognition signals were partially restored by applying the cuticular extracts on the elytra of rinsed female cadavers. No differences were observed in the male mating behavior responses to female cadavers reconstituted with their own crude extract and to freeze-killed females that were not solvent extracted. All 19 *H. betulinus* males made antennal contact with the female cadavers with the crude extract reapplied to the elytra and 15 of these males mounted the cadavers, rotated 180° and attempted copulation compared to 19 of 19 males to the freeze-killed females ($p = 0.105$, $n = 19$) (Table 1).

Even though the extraction and reapplication process might have changed the natural structure of the wax layer of the female epicuticle (see Ginzel et al. 2003; Barbour et al. 2007; Hughes et al. 2011), those changes did not significantly affect mate recognition by *H. betulinus* males. The absence of a mating behavior response of males to the solvent extracted females followed by males attempting copulation with rinsed female cadavers reconstituted with the crude extract demonstrates that some specific compound or combinations of compounds in the epicuticular wax layer of female *H. betulinus* are necessary for mate recognition in *H. betulinus*.

Table 1 Comparison of *Hedypathes betulinus* male copulation response to untreated freeze-killed conspecific female cadavers and male copulation response to female cadavers submitted to treatments (rinsed and sequentially reconstituted with the crude extract, individual fractions, binary or tertiary blends of fractions on the cuticle) using Fisher Exact Test (experiment wise-error = .05)

Bioassays	*p* values
Fraction E	1.839×10^{-4}*
Fraction H	7.708×10^{-7}*
Fraction C	3.351×10^{-9}*
Fractions E + H	2.113×10^{-2}*
Fractions H + C	2.405×10^{-6}*
Fractions E + C	1.453×10^{-4}*
Fractions E + H + C	4.359×10^{-4}*
Crude Extract	0.105

Fraction C Cyclohexene Fraction, *Fraction E* Ether Fraction, *Fraction H* Hexane Fraction

*significant values

Bioassays of Fractions

Ten *H. betulinus* female cadavers were treated with the tertiary blend of one FE of each of the ether fraction (Fraction E), cyclohexene fraction (Fraction C) and hexane fraction (Fraction H) to assay a cohort of 20 conspecific males (i.e., one treated female cadaver was assayed to two individual males). In total, ten positive responses were observed to the tertiary blend. A significant difference in male copulation response to untreated female cadavers and solvent extracted female cadavers treated with Fraction E, C and H was observed ($p = 4.359 \times 10^{-4}$, $n = 20$) (Table 1).

The mating response of cohorts of *H. betulinus* males (one cohort of 19 males, one cohort of 20 males and one cohort of 21 males) to female cadavers treated with a blend of one FE of each of the binary combinations (10 females treated with H + C, 10 females treated with E + C and 11 females treated with H + E) (each treated female cadaver was presented to two individual males) was observed. Of the 20 *H. betulinus* males assayed to female cadavers treated with one FE of Fraction C and E, nine males mounted, rotated 180° and attempted copulation. Male copulation response to untreated female cadavers and to female cadavers treated with Fraction C and E was significantly different ($p = 1.453 \times 10^{-4}$, $n = 20$) (Table 1). Five of the 19 *H. betulinus* males assayed to female cadavers with one FE of Fraction C and H applied to the elytra displayed behaviors 2, 3 and 4. Male copulation response to untreated female cadavers and female cadavers treated with Fraction C and H was significantly different ($p = 2.405 \times 10^{-6}$, $n = 19$). The highest number of positive male responses to female cadavers treated with binary extract combinations was observed to female cadavers treated with the binary blend of one FE of Fraction E and H. Sixteen of the 21 males assayed mounted, rotated 180° and attempted copulation with the female cadavers treated with Fraction E and H. Male copulation response to untreated female cadavers and female cadavers treated with Fraction E and H was significantly different ($p = 2.113 \times 10^{-2}$, $n = 21$) (Table 1).

Twenty *H. betulinus* males ($n = 20$) were assayed to female cadavers treated with one female equivalent (FE) of Fraction H (10 female cadavers). Another cohort of 20 *H. betulinus* males ($n = 20$) were assayed to female cadavers treated with one FE of Fraction C (10 female cadavers). A final cohort of 22 males ($n = 22$) were assayed to female cadavers treated with one

FE of Fraction E (11 female cadavers). One treated female cadaver was assayed to two individual males. Only two of 20 *H. betulinus* males displayed behaviors 2, 3 and 4 of the mating sequence to female cadavers treated with one FE of Fraction C. Significantly fewer males exhibited a copulation response to female cadavers treated with Fraction C than to untreated female cadavers ($p = 3.351 \times 10^{-9}$, $n = 20$) (Table 1). Five of the 20 *H. betulinus* males introduced to female cadavers treated with one FE of Fraction H mounted, rotated 180° and attempted copulation with the treated female cadavers. Male copulation response to untreated female cadavers and female cadavers treated with Fraction H were significantly different ($p = 7.708 \times 10^{-7}$, $n = 20$) (Table 1). The highest number of copulation attempts observed to the individual fractions was observed to females treated with Fraction E. Eleven of 22 males displayed behaviors 2, 3 and 4 to female cadavers treated with one FE of the Fraction E. Male copulation response to untreated female cadavers and female cadavers treated with Fraction E was significantly different ($p = 1.839 \times 10^{-4}$, $n = 22$) (Table 1).

The rate of male copulation response to female cadavers treated with the crude extract and the individual fractions and binary and tertiary blends of fractions were compared to determine the minimum number of fractions necessary and sufficient to recover the male copulation activity observed to the crude extract. Copulation response of males to fractions was compared with the copulation response of males to female crude extract instead of untreated freeze-killed females because the extraction of cuticular compounds from females and the application of the resulting crude extract back onto the solvent-rinsed cadaver alter the organization that these compounds are present in the cuticular wax layer and decrease the abundance of the contact pheromone on the surface of the female (Hughes et al. 2011). Twenty-eight paired contrasts of bioassays were done in total, but we are concentrating our discussion on 22 of the 28 paired contrasts since differences were observed in copulation response of males as the complexity of fraction blends increase (Table 2) (SM1). The more polar compounds of the female cuticle (Fraction E) are sufficient and necessary to stimulate the mating sequence in male *H. betulinus* ($p = 0.102$; comparison between male copulation response to female cadavers reconstituted with the crude extract and rinsed female cadavers treated with Fraction E) (Fig. 1).

Fraction C or H alone were not sufficient to stimulate male mating behavior response comparable to the crude extract of females [comparison between male copulation response to female cadavers reconstituted with the crude extract and rinsed female cadavers treated with Fraction H ($p = 1.231 \times 10^{-3}$) and Fraction C ($p = 1.664 \times 10^{-5}$)], but these fractions elicit male mating behavior response comparable to the crude extract when combined with Fraction E in binary ($p = 0.726$; comparison between male copulation response to female cadavers reconstituted with the crude extract and rinsed female cadavers treated with Fractions E + H) or tertiary ($p = 9.584 \times 10^{-2}$; comparison between male copulation response to female cadavers reconstituted with the crude extract and rinsed female cadavers treated with Fractions E + H + C) blends.

Analyses of Male and Female Cuticular Extracts and Fractions

Gas chromatographic analyses of cuticular extracts in double distilled hexane of male and female *H. betulinus*

Table 2 Paired contrasts of the rate of male copulation response using Fisher Exact Test (experiment wise-error = .05)

Bioassays	Crude Extract	Fraction E	Fraction H	Fraction C
Fraction E	0.102	–	–	–
Fraction H	1.231×10^{-3}*	0.121	–	–
Fraction C	1.664×10^{-5}*	7.410×10^{-3}*	0.407	–
Fractions E + H	0.726	0.215	4.804×10^{-3}*	4.482×10^{-5}*
Fractions H + C	2.918×10^{-3}*	0.199	1	0.235
Fractions E + C	4.804×10^{-2}*	0.767	0.320	3.095×10^{-2}*
Fractions E + H + C	9.584×10^{-2}	1	0.190	1.381×10^{-2}*

Fraction C Cyclohexene Fraction, *Fraction E* Ether Fraction, *Fraction H* Hexane Fraction

*significant *p* values

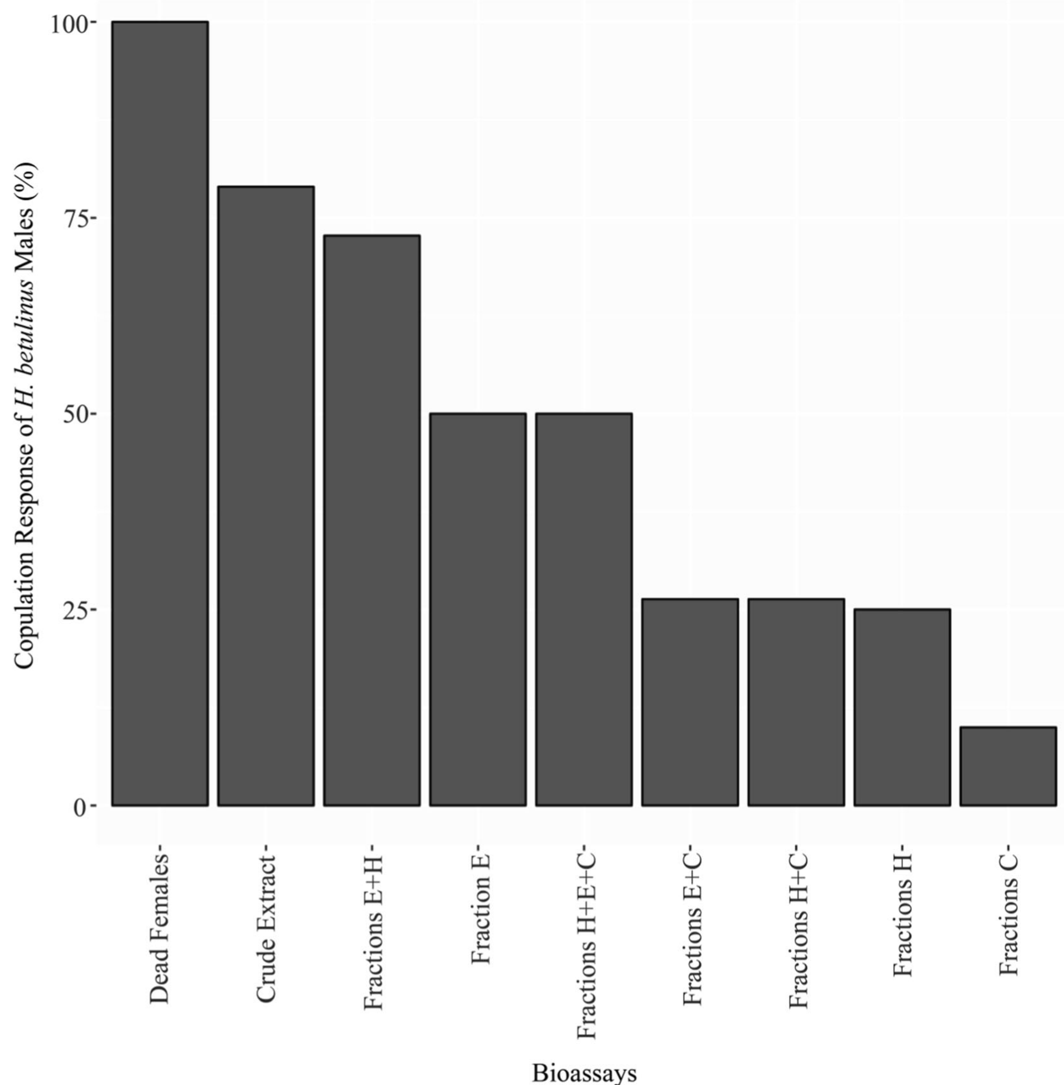

Fig. 1 The percentage of male *Hedypathes betulinus* that attempted copulation with freeze killed females with the cuticular profile intact (Dead Females), reconstituted female cadavers (Crude Extract Bioassay), and with female cadavers treated with individual fractions, binary and tertiary blends of fractions. Fraction C = Cyclohexene Fraction; Fraction E = Ether Fraction; Fraction H = Hexane Fraction

identified quantitative differences and two sex-specific compounds were identified in male cuticular extracts (Fig. 2). Comparison of GC-MS analyses of synthetic standards and these two compounds confirmed that compound 1 is (*E*)-6,10-dimethyl-5,9-undecadien-2-one (geranylacetone) (SM2) and compound 2 is (*R*)-(−)-(*E*)-6,10-dimethyl-5,9-undecadien-2-yl acetate (fuscumol acetate) (SM3). These compounds have been previously identified as being part of the long-range sex-pheromone of *H. betulinus* (Fonseca et al. 2010; Vidal et al. 2010). The combination of crude extracts of a cohort of five males and crude extracts of a cohort of five females and the fractionation of these male and female extracts with hexane, cyclohexene and ether facilitated clearer examination of the cuticular profile of male and female *H. betulinus* due to the higher concentration of compounds and partitioning of crude extracts in different fractions.

Quantitative differences in male and female fractions observed for the concentrations of two unidentified compounds from Fraction E (compounds Y and Z) were statistically different. Compound Y was present at an

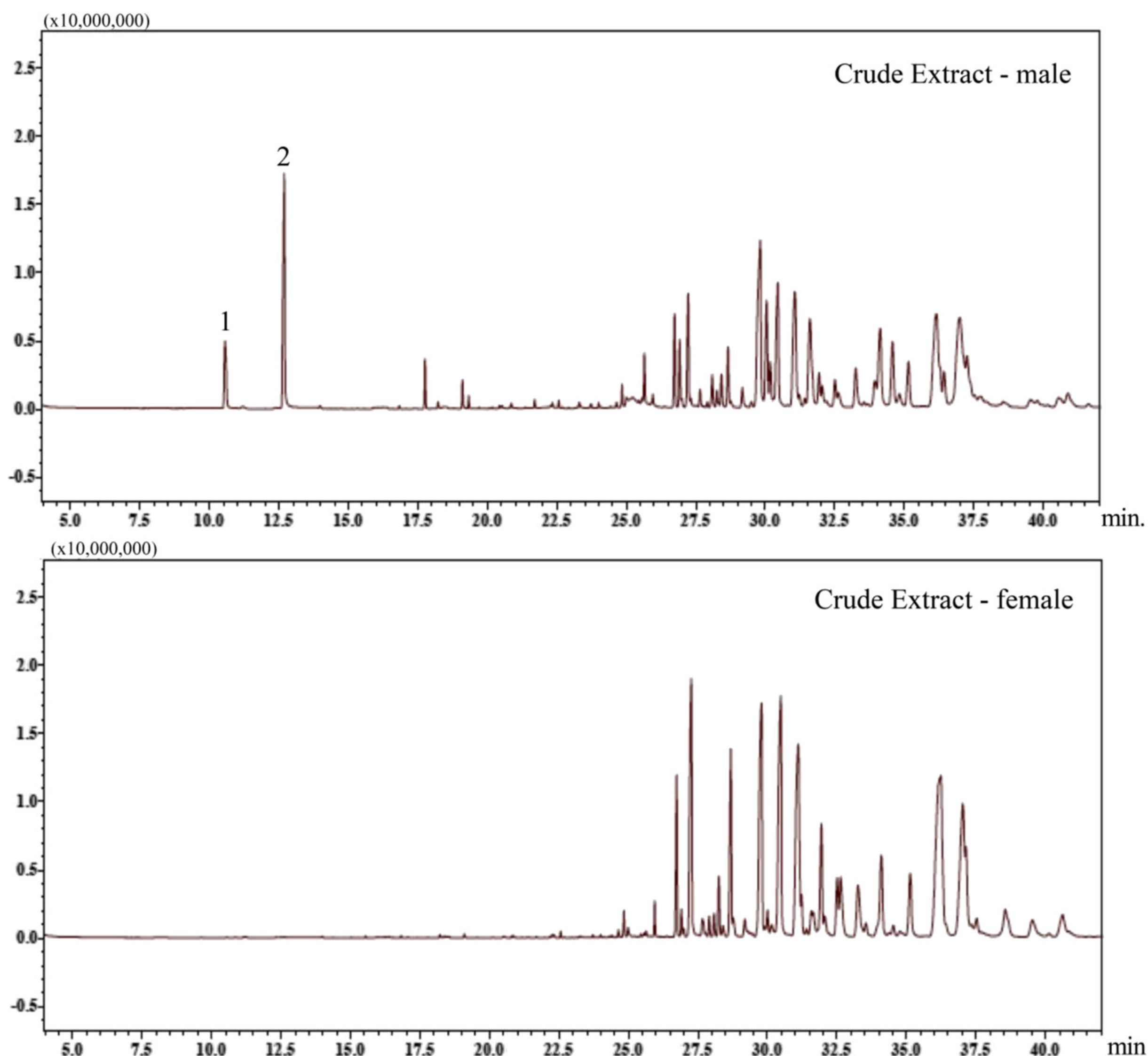

Fig. 2 Chromatograms of *Hedypathes betulinus* male and female cuticular extracts. 1 - (*E*)-geranylacetone; 2 - (*E*)-(*R*)-fuscumol acetate

average concentration of 2.18 ppm and 6.23 ppm (both 86.5 μL) of the Ether Fraction of one FE and ME, respectively (SHAPIRO-WILK, $p = 3.044 \times 10^{-2}$) (WILCOX, $z = -2.654759$, $p = 7.937 \times 10^{-3}$, $n = 5$). Compound Z was present at an average concentration of 4.36 ppm in one FE (86.5 μL) and 10.68 ppm in one ME (86.5 μL) of the ether faction (SHAPIRO-WILK, $p = 1.738 \times 10^{-1}$) (T-TEST, $t = -2.7677$, $p = 3.661 \times 10^{-2}$, $n = 5$) (SM4). Although compounds Y and Z were not identified, their mass spectra suggest that the compound Y might be a Δ-lactone (SM5), due to the fragmentation peak 99, and that compound Z might be a ketone (SM6).

Comparison of chromatograms of the ether fraction of male and female *H. betulinus* identified one compound present in very different concentrations in male and female fractions (SM4). This compound was identified as (*R*)-(*E*)-fuscumol acetate by comparison of the mass-spectra with the literature (SM7) and co-injection with synthetic standards (SM8, 9). Quantification of male ($n = 5$) and female ($n = 5$) ether fractions show that one FE of the female ether fraction (86.5 μL) had an average of 0.147 ppm of this compound, while one ME of the male ether fraction (86.5 μL) has an average of 29 ppm (SHAPIRO-WILK, $p = 1.218 \times 10^{-4}$) (WILCOX, $z = -2.411822$, $p = 1.587 \times 10^{-2}$, $n = 5$).

Synthetic Fuscumol Acetate Bioassays

Twenty-one *H. betulinus* males were assayed with 11 female cadavers treated with one FE of synthetic (*R/S*)-fuscumol acetate each. Of the 21 that made antennal contact with the treated female cadavers, only four males mounted, rotated 180° and attempted copulation. Male copulation response differed between untreated female cadavers and female cadavers treated with the FE of synthetic (*R/S*)-fuscumol acetate ($p = 4.700 \times 10^{-8}$, $n = 21$) (Table 3).

Eleven *H. betulinus* female cadavers treated with one FE of (*R*)-fuscumol acetate were introduced to 21 conspecific males. Five of the 21 males assayed mounted, rotated 180° and attempted copulation with the 11 female cadavers treated with FE of (*R*)-fuscumol acetate. A significant difference in male copulation response to untreated female cadavers and solvent extracted female cadavers treated with the FE of (*R*)-fuscumol acetate was observed ($p = 2.444 \times 10^{-7}$, $n = 21$). Twenty *H. betulinus* males were assayed to 10 conspecific female cadavers treated with one FE of (*S*)-fuscumol acetate. Of the 20 males that made antennal contact with the treated female cadavers, only three males mounted, rotated 180° and attempted copulation with treated female cadavers. Male copulation response to untreated female cadavers differed from male response to female cadavers treated with a FE of (*S*)-fuscumol acetate ($p = 2.569 \times 10^{-8}$, $n = 20$). Ten *H. betulinus* female cadavers treated with one ME of (*R*)-fuscumol acetate were introduced to 19 conspecific males. One of the 19 males assayed mounted, rotated 180° and attempted copulation with the 10 female cadavers treated with one ME of (*R*)-fuscumol acetate. Significantly fewer males exhibited a copulation response to female cadavers treated with the ME of (*R*)-fuscumol acetate than to untreated female cadavers ($p = 3.390 \times 10^{-5}$, $n = 19$) (Table 3) (Fig. 3).

Table 3 Comparison of *Hedypathes betulinus* male copulation response to untreated freeze-killed conspecific female cadavers and male copulation response to female cadavers submitted to treatments (rinsed and sequentially reconstituted with one female equivalent (FE) of the synthetic (*R/S*)-, (*R*)- or (*S*)-fuscumol acetate or one male equivalente (ME) of the synthetic (*R*)-fuscumol acetate on the cuticle) using Fisher Exact Test (experiment wise-error = .05)

Bioassays	*p* values
FE (*R/S*)-Fuscumol Acetate	4.700×10^{-8}*
FE (*R*)-Fuscumol Acetate	2.444×10^{-7}*
FE (*S*)-Fuscumol Acetate	2.569×10^{-8}*
ME (*R*)-Fuscumol Acetate	1.131×10^{-9}*

*significant values

Comparison of Synthetic Fuscumol Acetate and the Female Ether Fraction

The response of male *H. betulinus* to female cadavers treated with one FE of Fraction E ($n = 22$) and female cadavers treated with one FE of the *R* enantiomer of fuscumol acetate ($n = 21$) did not differ ($p = 0.115$). In addition, no differences were observed between male *H. betulinus* response to female cadavers treated with one FE of Fraction E ($n = 22$) and female cadavers treated with one FE of (*R/S*)-fuscumol acetate ($n = 21$) ($p = 5.461 \times 10^{-2}$) (Table 4).

Discussion

Studies of the role of cuticular hydrocarbons in mate recognition systems of insects offer important insights about how differences in the cuticular chemical profile are involved in mating behavior and success (Chung and Carroll 2015). Many studies have proven that chemical compounds present on the body surface of females are important for mate recognition in Cerambycidae (Kim et al. 1993; Ginzel et al. 2003; Ginzel and Hanks 2003, 2005; Barbour et al. 2007; Mohd Sabri and Abdullah 2016), but only a few components of active cuticular sex-pheromones have been identified. Millar and Hanks (2017) suggest that this might be related to the structure of cuticular lipids, which are very complex, and the difficulty in purifying and obtaining individual standards for tests in bioassays. This study provides additional knowledge of the role of female contact pheromones in the mating behavior of *H. betulinus* and confirms that males rely on compounds present in the epicuticular wax layer of females for mate recognition.

A previous study determined that males of *H. betulinus* would only attempt copulation with females after antennal contact was made and solvent extraction of freeze-killed female cadavers made them unattractive to conspecific males in laboratory assays (Fonseca and Zarbin 2009). In our study, mate recognition was partially restored by re-applying the cuticular compounds on the elytra of rinsed female cadavers. However, there is a possibility that some chemical

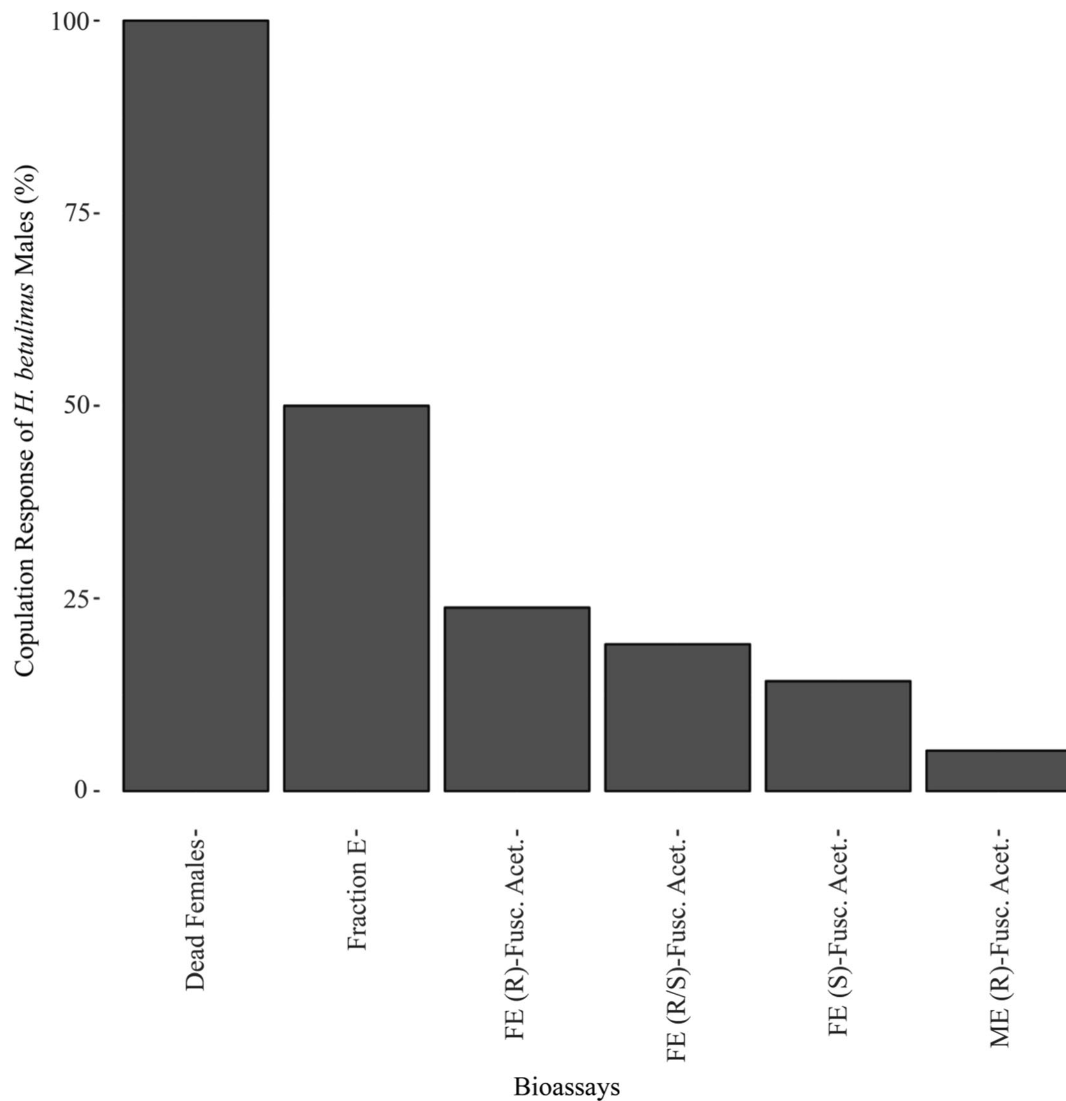

Fig. 3 The percentage of male *Hedypathes betulinus* that attempted copulation with freeze-killed females with the cuticular profile intact (Dead Females) and with solvent-extracted female cadavers treated with individual ether fraction (Fraction E) and synthetic (*R,S*)-, (*R*)- or (*S*)-fuscumol acetate. FE (R)-Fusc. Acet. = female equivalent of synthetic (*R*)-fuscumol acetate; FE (R/S)-Fusc. Acet. = female equivalent of synthetic (*R/S*)-Fuscumol Acetate; FE (S)-Fusc. Acet. = female equivalent of synthetic (*S*)-fuscumol acetate; ME (R)-Fusc. Acet. = male equivalent of synthetic (*R*)-fuscumol acetate

compounds present in the epicuticular wax layer of *H. betulinus* females that play a role in the mate recognition system of this species were lost or altered during the extraction, fractionation and reapplication process (Ginzel et al. 2003; Barbour et al. 2007; Fonseca and Zarbin 2009; Hughes et al. 2011).

The compounds necessary and sufficient to stimulate male copulation response in *H. betulinus* are present in the more polar fraction of female extracts (Fraction E). Fukaya et al. (1999, 2000) reported that the female contact sex pheromone of *Anoplophora malasiaca* (Cerambycidae: Lamiinae) consists of saturated hydrocarbons and more polar compounds. Yasui et al. (2003, 2007) identified the female contact sex pheromone of *A. malasiaca* as a mixture of four methyl-branched alkanes (9-methylheptacosane, 9-methylnonacosane, 15-methylhentriacontane and 15-methyltritriacontane), four ketones (heptacosan-10-

Table 4 Paired contrasts of the male copulation response of all synthetic fuscumol acetate bioassays to the male copulation response rate of female ether fraction bioassay using Fisher Exact Test (experiment wise-error = .05)

Bioassays	F Fraction E	FE (*R*)-Fusc. Acet.	FE (*S*)-Fusc. Acet.	FE (*R/S*)-Fusc. Acet.
FE (*R*)-Fusc. Acet.	0.115	–	–	–
FE (*S*)-Fusc. Acet.	2.320×10^{-2}*	0.696	–	–
FE (*R/S*)-Fusc. Acet.	5.461×10^{-2}	1	1	–
ME (*R*)-Fusc. Acet.	1.995×10^{-3}*	0.185	0.604	0.345

F Fraction E female ether fraction, *FE* female equivalent, *ME* male equivalent, *Fusc. Acet.* synthetic fuscumol acetate

*significant *p* values

one, (*Z*)-18-heptacosen-10-one, (18*Z*,21*Z*)-heptacosa-18,21-dien-10-one and (18*Z*,21*Z*,24*Z*)-heptacosa-18,21,24-trien-10-one) and three complex bicyclic lactones.

Gas chromatography analyses of cuticular profiles of some Cerambycidae species have shown few differences between males and females (see Millar and Hanks 2017) despite the fact that males can clearly differentiate other males from females after antennal contact is made (Hanks et al. 1996). Our study shows that the compound with the largest difference in concentration between male and female *H. betulinus* in Fraction E is (*E*)-(*R*)-fuscumol acetate, the male produced sex-aggregation pheromone (Fonseca et al. 2010; Vidal et al. 2010). The low concentrations observed in female cuticular extracts may indicate that females produce (*E*)-(*R*)-fuscumol acetate in very low concentrations, or it could be transferred from males to females during copulation (adult females used in the laboratory bioassays were collected from the field and may have interacted with males prior to assay). Fonseca et al. (2010) detected a larger amount of total volatiles released by male *H. betulinus* that were fed with green mate twigs than males that did not feed on green mate. This result suggests a relationship between the production of pheromone by *H. betulinus* and diet (Fonseca et al. 2010). Geranylacetone is found in large amounts in the essential oil of leaves of green mate (Bastos et al. 2006) and it is the precursor in the biosynthesis of fuscumol and fuscumol acetate (Fonseca et al. 2010). Male *H. betulinus* might sequester this compound from green mate during feeding and use it in pheromone production (Zarbin et al. 2013). There is evidence that diet has an impact on cuticular hydrocarbon composition of laboratory colonies of *Acanthoscelides obtectus* (Coleoptera: Chrysomelidae) in which the levels of three hydrocarbon compounds present in the cuticular profile of females (*n*-pentacosane, 9-methylheptacosane, and *n*-triacontane) vary between two female populations who fed on either beans or chickpeas for 50 generations, and this difference could be detected by males who were reared on chickpeas for 50 generations (Stojković et al. 2014).

Limitations of this study include the fact that all insects used in the laboratory bioassays were collected from the field and as a result we were not able to determine their exact age and mating status. Insect cuticular profiles have been reported to differ among individuals of different ages (Kuo et al. 2012), between maturation-fed and unfed females (Brodie et al. 2012), and between virgin and mated females (Carlson and Schlein 1991). In addition, all females used in this study were killed by freezing prior to assay, which could have eliminated tactile or behavioral stimuli involved in mate recognition of *H. betulinus* (see Fonseca and Zarbin 2009). As an independent test, a glass rod treated with one FE of crude extract was presented to two individual males. Both males assayed with the female extract treated glass rod attempted to copulate with it, suggesting that males rely on chemical rather than behavioral or visual stimuli to identify conspecific females. However, more assays are necessary to confirm that male *H. betulinus* do not use behavioral and tactile signals in mate recognition.

This study demonstrates that contact pheromones mediate mate recognition and mating behavior in *H. betulinus*. The more polar compounds present in the ether fraction of female extracts are necessary and sufficient to recover male copulation response to rinsed female cadavers and their interaction with saturated and unsaturated hydrocarbons of females might be important for mate recognition in this species. Future studies should concentrate on determining if (*E*)-(*R*)-fuscumol acetate is produced by female *H. betulinus* or if it could be transferred from males after copulation. In addition, further investigation of the saturated and unsaturated hydrocarbons, and

more polar compounds present in the epicuticular wax layer of female *H. betulinus* are necessary in order to identify additional components of the contact pheromone of this species.

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Compliance with Ethical Standards

Conflict of Interest The authors state that there are no conflicts of interest.

References

Allison JD, Borden JH, Seybold SJ (2004) A review of the chemical ecology of the Cerambycidae (Coleoptera). Chemoecology 14:123–150

Barbour JD, Lacey ES, Hanks LM (2007) Cuticular hydrocarbons mediate mate recognition in a species of longhorned beetle (Coleoptera: Cerambycidae) of the primitive subfamily Prioninae. Ann Entomol Soc Am 100:333–338

Bastos DHM, Ishimoto EY, Marques MOM, Ferri AF, Torres EA (2006) Essential oil and antioxidant activity of green mate and mate tea (*Ilex paraguariensis*) infusions. J Food Compos Anal 19:538–543

Brodie BS, Wickham JD, Stephen AT (2012) The effect of sex and maturation on cuticular semiochemicals in *Monochamus scutellatus* (Coleoptera: Cerambycidae). Can Entomol 144: 826–833

Cardé RT (2014) Defining attraction and aggregation pheromones: teleological versus functional perspectives. J Chem Ecol 40: 519–520

Carlson DA, Schlein Y (1991) Unusual polymethyl alkenes in tsetse flies acting as abstinon in *Glossina morsitans*. J Chem Ecol 17:267–284

Cassanello AML (1993) Ciclo de Vida e aspectos morfológicos de *Hedypathes betulinus* (Klug, 1825) (Coleoptera, Cerambycidae, Lamiinae), broca-da-erva-mate (*Ilex paraguariensis* St. Hil.). Thesis, Universidade Federal do Paraná

Chung H, Carroll SB (2015) Wax, sex and the origin of species: dual roles of insect cuticular hydrocarbons in adaptation and mating. Bioessays 37:822–830

EMBRAPA, Sistemas de Produção Embrapa. Produção de Erva-Mate (2010) https://www.spo.cnptia.embrapa.br/. Accessed 18 Apr 2018

Fonseca MG, Zarbin PHG (2009) Mating behaviour and evidence for sex-specific pheromones in *Hedypathes betulinus* (Coleoptera: Cerambycidae: Lamiinae). J Appl Entomol 133:695–701

Fonseca MG, Vidal DM, Zarbin PH (2010) Male produced sex-pheromone of the Cerambycidae beetle *Hedypathes betulinus*: chemical identification and biological activity. J Chem Ecol 36:1136–1139

Fukaya M, Akino T, Yasuda T, Tatsuki S, Wakamura S (1999) Mating sequence and evidence for synergistic component in female contact sex pheromone of the white-spotted longicorn beetle, *Anoplophora malasiaca* (Thomson) (Coleoptera: Cerambycidae). Entomol Sci 2:183–187

Fukaya M, Akino T, Yasuda T, Wakamura S, Satoda S, Senda S (2000) Hydrocarbon components in contact sex pheromone of the whitespotted longicorn beetle, *Anoplophora malasiaca* (Thomson) (Coleoptera: Cerambycidae) and pheromonal activity of synthetic hydrocarbons. Entomol Sci 3:211–218

Ginzel MD, Hanks LM (2003) Contact pheromones as mate recognition cues of four species of longhorned beetles (Coleoptera: Cerambycidae). J Insect Behav 16:181–187

Ginzel MD, Hanks LM (2005) Role of host plant volatiles in mate location for three species of longhorned beetles. J Chem Ecol 31:213–217

Ginzel MD, Blomquist GJ, Millar JG, Hanks LM (2003) Role of contact pheromones in mate recognition in *Xylotrechus colonus*. J Chem Ecol 29:533–545

Ginzel MD, Moreira JA, Ray AM, Millar JG, Hanks LM (2006) (Z)-9-Nonacosene—major component of the contact sex pheromone of the beetle *Megacyllene caryae*. J Chem Ecol 32:435–451

Guedes JVC, D'avila M, SHB D (2000) Comportamento de *Hedypathes betulinus* (Klug, 1825) em erva-mate em campo. Ciênc Rural 30:1059–1061

Hanks LM (1999) Influence of the larval host plant on reproductive strategies of cerambycid beetles. Annu Rev Entomol 44: 483–505

Hanks LM, Millar JG (2016) Sex and aggregation-sex pheromones of cerambycid beetles: basic science and practical applications. J Chem Ecol 42:631–654

Hanks LM, Millar JG, Paine TD (1996) Mating behavior of the eucalyptus longhorned borer (Coleoptera: Cerambycidae) and the adaptive significance of long "horns". J Insect Behav 9:383–393

Hughes GP, Spikes AE, Holland JD, Ginzel MD (2011) Evidence for the stratification of hydrocarbons in the epicuticular wax layer of female *Megacyllene robiniae* (Coleoptera: Cerambycidae). Chemoecology 21:99–105

IBGE, Diretoria de Pesquisas, Coordenação de Agropecuária. Produção da Extração Vegetal e da Silvicultura (2016) https://www.ibge.gov.br/estatisticas-novoportal/economicas/agricultura-epecuaria/9105-

producao-da-extracao-vegetal-e-da-silvicultura.html?=&t=resultados. Accessed 28 Mar 2018

Iede ET, Machado DC (1989) Pragas da erva-mate (*Ilex paraguariensis* St. Hill.) e seu controle. Embrapa Florestas-Artigo em Periódico Indexado (ALICE). http://www.alice.cnptia.embrapa.br/alice/handle/doc/282097. Accessed 24 July 2018

Kim GH, Takabayashi J, Takahashi S, Tabata K (1993) Function of contact pheromone in the mating behavior of the cryptomeria bark borer, *Semanotus japonicus* Lacordaire (Coleoptera: Cerambycidae). Appl Entomol Zool 28:525–535

Kuo TH, Yew JY, Fedina TY, Dreisewerd K, Dierick HA, Pletcher SD (2012) Aging modulates cuticular hydrocarbons and sexual attractiveness in *Drosophila melanogaster*. J Exp Biol 215:814–821

Li TS, Li JT, Li HZ (1995) Modified and convenient preparation of silica impregnated with silver nitrate and its application to the separation of steroids and triterpenes. J Chromatogr A 715:372–375

Millar JG, Hanks LM (2017) Chemical ecology of Cerambycidae. In: Wang Q (ed) Cerambycidae of the world: biology and Pest management. CRC Press, Boca Raton, pp 161–208

Mohd Sabri MS, Abdullah F (2016) Mating behaviour and evidence of a female sex pheromone in *Rytidodera simulans* white (Coleoptera: Cerambycidae). J Entomol Res 40:313–326

Penteado SRC (1995) Principais pragas da erva-mate e medidas alternativas para seu controle. In: Winge H, Ferreira AG, Mariath JEA, Tarasconi LC (eds) Erva-mate: Biologia e Cultura no Cone, Sul. edn. Universidade, Porto Alegre, pp 109–102

Stojković B, Savković U, Đorđević M, Tucić N (2014) Host-shift effects on mating behavior and incipient pre-mating isolation in seed beetle. Behav Ecol 25:553–564

Vidal DM, Fonseca MG, Zarbin PH (2010) Enantioselective synthesis and absolute configuration of the sex pheromone of *Hedypathes betulinus* (Coleoptera: Cerambycidae). Tetrahedron Lett 51:6704–6706

Yasui H, Akino T, Yasuda T, Fukaya M, Ono H, Wakamura S (2003) Ketone components in the contact sex pheromone of the whitespotted longicorn beetle, *Anoplophora malasiaca*, and pheromonal activity of synthetic ketones. Entomol Exp Appl 107:167–176

Yasui H, Akino T, Yasuda T, Fukaya M, Wakamura S, Ono H (2007) Gomadalactones a, B, and C: novel 3-oxabicyclo[3.3.0]octane compounds in the contact sex pheromone of the white-spotted longicorn beetle, *Anoplophora malasiaca*. Tetrahedron Lett 48:2395–2400

Zarbin PH, Fonseca MG, Szczerbowski D, Oliveira AR (2013) Biosynthesis and site of production of sex pheromone components of the cerambycid beetle, *Hedypathes betulinus*. J Chem Ecol 39:358–363

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