Mating behaviour and evidence for sex-specific pheromones in *Hedypathes betulinus* (Coleoptera: Cerambycidae: Lamiinae)

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Abstract

The mating behaviour and evidence for sex-specific pheromones of the main pest of green mate, *Ilex paraguariensis*, of the southern region of Brazil, *Hedypathes betulinus* (Klug, 1825) (Coleoptera: Cerambycidae: Lamiinae), were studied in the laboratory. The mating sequence of *H. betulinus* includes: female approaching the male, antennal contact, male mounting the female and start of copulation. This mating sequence provided support for our hypothesis that recognition of males by females may be mediated by a male-produced pheromone and males recognize females by contact pheromones. The behavioural responses in an olfactometer of male and female *H. betulinus* to volatiles released by male and female conspecifics confirmed the presence of a male-produced sex pheromone. This finding suggests that visual cues are not primary in eliciting a sexual response from females. Three male-specific compounds were detected in the chromatographic analysis, providing chemical support to the behavioural data. Males did not respond to dead-washed females but 70% of males attempted to mate with dead-washed extract-treated females, suggesting that extracts contained contact pheromones. Based on these results, a male-produced sex pheromone and a female-produced contact pheromone should be essential in the communication system of *H. betulinus*.

Introduction

Cerambycid beetles are among the most important insect pests worldwide, degrading lumber and damaging and killing trees in forests, orchards, plantations and urban landscapes. The lack of information on behaviour of adults, particularly the role of semiochemicals in reproduction, hinders development of effective detection and management strategies for many cerambycid species that are economically important pests (Solomon 1995).

In most cerambycid beetles, it has been considered that mate location depends on males encountering females by chance, and that recognition of females is by antennal contact or in response to very short-range pheromones that only operate over distances of several centimetres (Hanks 1999). In some cerambycids, there is evidence of volatile pheromones that act over short and/or long distances for mate location (Schlyter and Birgersson 1999; Allison et al. 2004).

Green mate, *Ilex paraguariensis* St. Hil., is the economic base of several municipalities in the southern region of Brazil. One of the biggest problems faced by its producers is the control of insect pests, being *Hedypathes betulinus* (Klug, 1825) the main one (Guedes et al. 2000).

The adults measure approximately 25 mm of length and have white coloured body with dark spots; they present long and thin antennae with alternated dark and white spots. During oviposition, the female places only one egg in each branch. The larvae are white, apod and bore into and feed on the branches and trunks of green mate. They build longitudinal galleries into the branches, which stop the normal circulation of the sap, resulting in mori-

bund plants. As the bore progresses, the larvae leave sawdust behind it, which accumulates in the base of the stalk, accusing its presence. The larvae can destroy 60% of green mate plants (Brandão Filho 1945; Mazuchowski 1991; Cassanello 1993), therefore, two management strategies, collecting manually the adults and uprooting infested branches and trunks, intend to prevent infestation.

Knowledge of *H. betulinus* mating behaviour may provide information for the development of monitoring and control measures. This study was undertaken to investigate mating behaviour sequence and to search for sex-specific pheromones in *H. betulinus*.

Materials and Methods

Insects

Adult insects used in all experiments were collected directly from green mate crops located in São Mateus do Sul, Parana, Brazil in December 2006 through May 2007 and transferred to the laboratory. They were sexed according Cassanello (1993), who described that males have thicker antennae scape and femur fore legs than females. Males and females were held separately in plastic boxes (7.5 cm diameter × 8.5 cm high), were fed with green mate branches, and maintained at 25 ± 2°C, 60 ± 5% RH and a photoperiod of 12 : 12 L : D. The mating history of field-captured beetles was unknown.

Mating behaviour

In the field, *H. betulinus* mating has been observed during photophase, mainly between 2:00 and 6:00 p.m. (Guedes et al. 2000). To describe the mating behaviour sequence of *H. betulinus* and identify cues in mate location, we observed beetles in laboratory arenas during the same time period. Mating pairs (n = 8) were placed with sexes 30 cm apart in individual plastic containers (38 cm length × 27 cm width). For each pair, we recorded how sexes approached each other until the final mating. The mating behaviour of beetles was recorded using a digital camera (HP Photosmart M627; Hewlett-Packard, Miami, FL, USA) to facilitate analysis. We studied videotapes of beetles to determine whether one sex showed directed movements towards the other from a distance.

Collection of volatiles

Groups of four males and females, separated by sex, were placed in glass aeration chambers (33 cm height × 3.5 cm outside diameter) and volatiles emitted were trapped on 0.8 g of Super Q (Alltech, Deerfield, IL) columns as previously reported (Zarbin et al. 2003), and collected daily for 24 h over 15 consecutive days. A pushed humidified and charcoal filtered airstream (1 l/min) was maintained through the aeration apparatus. Each aeration chamber was connected to a glass adsorbent trap (11 cm long × 1 cm diameter) containing Super Q. Volatiles were eluted from Super Q with distilled hexane, changing the adsorbent traps each 10 collections. The daily extracts were not combined and were concentrated to 400 μl (one insect per 100 μl) under an argon stream (Zarbin et al. 1999; Zarbin 2001).

Olfactometer bioassay

Testing a male-produced sex pheromone

Response of *H. betulinus* to volatiles from either sex was tested in a binary choice Y-tube olfactometer, using humidified, charcoal filtered air at a rate of 4 l/min. Hydrochloric acid and ammonium hydroxide were mixed to visualize the plume distribution inside the system (Baker and Linn 1984). The olfactometer consisted of a Y-shaped glass tube 4 cm in diameter with a 40-cm long main tube and two 20-cm long arms. Odour sources consisted of a 2 × 2 cm piece of filter paper loaded with 20 μl of the extract (0.2 insect equivalent) of either male or female volatile extract or hexane (control), placed into the base of either arm of the olfactometer. One male or female was introduced into the base of the main tube of the olfactometer and their behaviour was observed for 20 min. Beetles that walked upwind and made direct contact with the filter paper that contained the odour source (insect extract or control) within 20 min was recorded as a response. A beetle that did not walk upwind to any odour source within 20 min was recorded as no response. After every four insects tested, the Y-tube was cleaned with alcohol and left to dry for 5 min and the positions of the olfactometer arms were inverted between odour sources to avoid any positional bias. Previous test showed that there was no difference in choice made either by male or female in the olfactometer when both of the arms were blank, indicating that there was no a positional bias in the olfactometer. Four experiments were conducted: (i) response of males to male extract vs. control; (ii) response of females to male extract vs. control; (iii) response of male to female extract vs. control; and (iv) response of female to female extract vs. control. For each experiment, we tested at least

50 individuals and each individual was tested once only. Experiments were performed during the fourth to 10th hour of photophase when adult beetles were typically active (Guedes et al. 2000).

Data on the response of males and females to different treatments (male extract, female extract vs. control) were analysed by the chi-squared test using BioEstat 3.0 (Ayres et al. 2003). Insects that did not make a choice were excluded from statistical analysis.

Analytical procedures

Gas chromatographic (GC) analyses were performed on a Varian 3800 GC equipped with flame ionization detector, electronic pressure control, and operated in splitless mode. A VA-5 (30 m × 0.25 m × 0.25 μm) capillary column was used under the following analytical conditions: initial temperature of 50°C for 1 min with an increase 7°C/min until 250°C, which was maintained for 10 min. Upon finishing, the chromatograms obtained with extracts of female and males were analysed for the presence of sex-specific candidate pheromone components.

Contact pheromone bioassay

This test was performed following the procedure described by Ginzel and Hanks (2003) and Ginzel et al. (2003). Individual females were freeze killed (−4°C for 30 min), allowed to warm to room temperature (∼15 min), and presented to a male in a plastic arena (15 cm diameter × 18 cm tall) to test whether males would recognize dead females and attempt to mate, demonstrating that recognition cues were intact. Subsequently, cuticular hydrocarbons of females were removed by immersing them in three 1-ml aliquots of hexane for 10 min each; aliquots were combined and concentrated to 1 ml under an argon stream. Dead-washed females were then presented individually to the same male to see if that male no longer responded, demonstrating that chemical cues had been removed and mating was not elicited by mechanoreception alone. To prove that the extract contained the pheromone, we pipetted 1 ml female extract on each original dead-washed female, allowed it to evaporate, and presented the female again to males to see if the recognition cue was restored. A trial was scored as a 'response' if the male, after antennal contact, mounted her and attempted to copulate. Non-response males showed none of theses behaviours or continued to walk after first contacting the female. Each of 10 dead females was presented to two different males (n = 20).

Responses of males to dead female and dead-washed extract-treated females were compared to their response to dead-washed females using a chi-squared test, program BioEstat 3.0 (Ayres et al. 2003).

Results and Discussion

The mating sequence of *H. betulinus* includes approach of the female to the male, antennal contact, mounting and copulation. All couples observed performed the following mating sequence: (i) Approach: Females within 30 cm of a males, always approached the motionless males, not the reverse (stages 1 and 2, fig. 1), suggesting that female was responding to either visual or chemical cues provided by the males.

No pre-mounting courtship display by *H. betulinus* males was observed (stages 1 and 2, fig. 1). Similarly

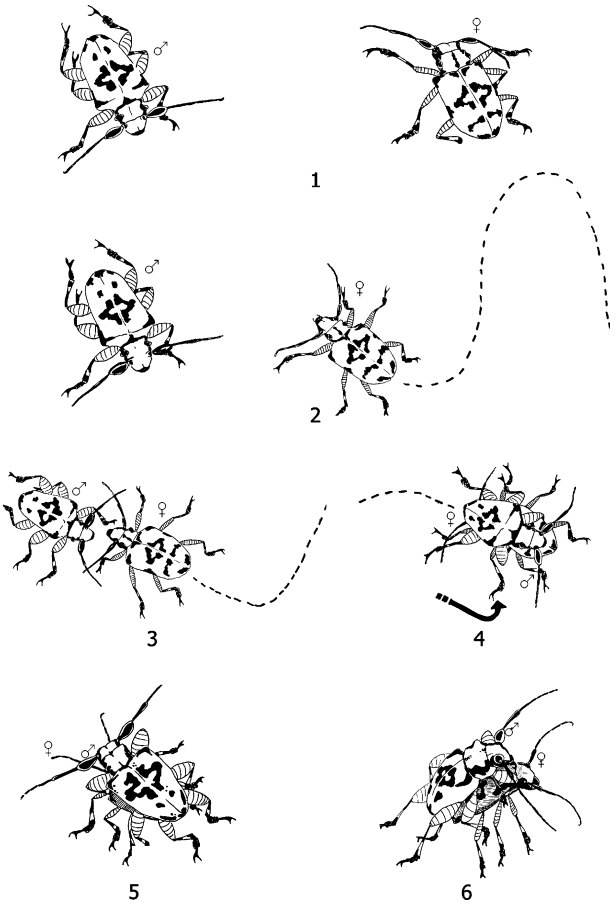

Fig. 1 Mating behaviour in *Hedypathes betulinus*. 1 – Female and male from distance of 30 cm; 2 – female approaches male; 3 – female touches male antennae with her antennae; 4 – male mounts the female, rotating 180°; 5 – male clasps the female pronotum or elytra with his forelegs; 6 – copulation.

to *Oemona hirta* (Wang and Davis 2005) and many other cerambycids (Hanks 1999). (ii) Antennal contact: the female walked towards the male and touched his antennae with her antennae (stage 3, fig. 1). (iii) Mounting: immediately after antennal contact, the male mounted the female, rotating 180°, and clasped her pronotum or elytra with his forelegs (stages 4 and 5, fig. 1). Males showed no interest in females until antennal contact, suggesting that chemical recognition of females is involved. (iv) Copulation: The male bends his abdomen to connect the female genitalia, and extracts her ovipositor by extending his hind legs (stage 6, fig. 1).

All females were receptive and usually immobile during copulation or slowly walked short distances and did not move their antennae. A similar behaviour was observed in *Neoclytus mucronatus mucronatus*, *Megacyllene caryae* and *Megacyllene robiniae* (Ginzel and Hanks 2003). After mating, males *H. betulinus* guarded females by remaining on their back. This behaviour was observed in the field for the same species by Guedes et al. (2000). Mate guarding is well known in lamiine cerambycids, including *Anoplophora chinensis* (Wang et al. 1996b) and *Anoplophora glabripennis* (Lance et al. 2003; Morewood et al. 2004). Male mate-guarding has usually been explained as competition for fertilization of more eggs by fending off rival males (Wang et al. 1996b; Hanks 1999; Alcock 2004).

These results of the mating sequence provided support for our hypothesis that recognition of males by females may be mediated by either a male-produced pheromone or vision and that males recognize females by contact pheromones.

In the Y-olfactometer bioassays, *H. betulinus* females were significantly attracted to odours from male volatile extract when compared to a hexane control: 36 females chose the male extract and eight females chose the control ($\chi^2 = 17.82$; d.f. = 1; $P < 0.01$); however, males were not attracted to odours from male extract: 18 males chose the male extract and 14 males chose the control ($\chi^2 = 0.50$; d.f. = 1; $P = 0.60$) (table 1). Neither females ($\chi^2 = 0.23$; d.f. = 1; $P = 0.75$) nor males ($\chi^2 = 0.39$; d.f. = 1; $P = 0.68$) were attracted to odours from female volatile extract (table 1). The attraction of only females to male volatile extract provides evidence that the mating sequence *H. betulinus* is initially mediated by a male-produced sex pheromone.

Sex pheromones are thought to be the most important cue for mate recognition in cerambycid beetles (Hanks 1999; Allison et al. 2004). For example, male-produced sex pheromones have been reported in *Monochamus alternatus* (Fauziah et al. 1987), *Anaglyptus subfasciatus* (Nakamuta et al. 1994), *Hylotrupes bajulus* (Fettköther et al. 1995), *Anoplophora glabripennis* (Zhang et al. 2002), *Steirastoma breve* (Liendo et al. 2005), *Anoplophora malasiaca* (Fukaya et al. 2005) and *Xylotrechus quadripes* (Hall et al. 2006). Silk et al. (2007) recently reported a male-produced pheromone in *Tetropium fuscum* and *Tetropium cinnamopterum*, the first evidence in the Spondylidinae subfamily.

Table 1 Responses of individual male and female *Hedypathes betulinus* adults to male/female extracts in Y-tube olfactometer

Odour sources	Sex	N	Response	Response (%) Extract	Hexane	P
Male extract vs. Hexane	Male	57	32	56.25	43.75	0.60
	Female	54	44	81.82	18.18	<0.00
Female extract vs. Hexane	Male	51	23	56.52	43.48	0.68
	Female	54	39	53.85	46.15	0.75

Statistically significant differences, chi-squared test, $P < 0.05$.

The chromatographic profiles of volatiles from male and female *H. betulinus* are shown in fig. 2, and clearly indicate the existence of three male-specific compounds (a–c), providing chemical support to the olfactometer bioassay data.

Furthermore, the mating sequence in *H. betulinus* demonstrated that no males of *H. betulinus* approached females directly until the latter touched them with their antennae (fig. 1). This reliance on antennal contact is apparently common among longhorned beetles (Michelsen 1966; Hughes 1981; Akutsu and Kuboki 1983; Kim et al. 1992; Hanks et al. 1996; Wang et al. 1996a; Lingafelter 1998; Dejia et al. 1999; Hanks 1999) and is consistent with recognition by contact pheromones (Kim et al. 1993; Fukaya et al. 1996, 2000; Wang 1998; Ginzel et al. 2003). In the same manner, the use of palpi in mating recognition is probably involved in mate recognition of some cerambycids (Fukaya and Honda 1992; Ibeas et al. 2008). For example, males of *M. alternatus* (Kim et al. 1992), *A. malasiaca* (Fukaya et al. 2000; Akino et al. 2001), *Psacothea hilaris* (Fukaya et al. 1996, 1997), *Xylotrechus colonus* (Ginzel et al. 2003), *Neoclytus mucronatus mucronatus*, *Megacyllene caryae*, *M. robiniae* (Ginzel and Hanks 2003), *Dectes texanus texanus* (Crook et al. 2004), *Prionus californicus* (Barbour et al. 2007), *Monochamus galloprovincialis* (Ibeas et al. 2008, 2009) display behaviour that suggest the use of contact pheromones.

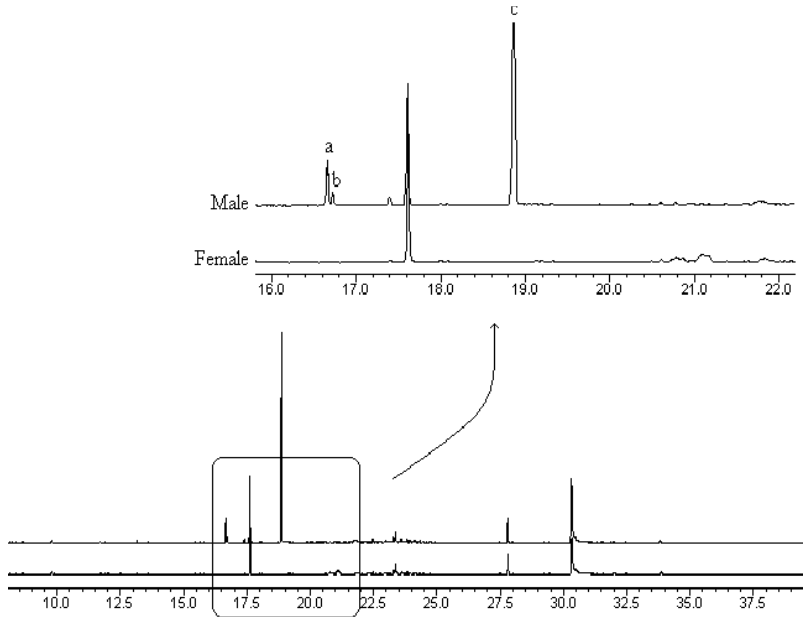

Fig. 2 Gas chromatographic analysis of volatiles obtained from male and female *Hedypathes betulinus* adults, showing three male-specific compounds, peaks a, b and c.

In the arena bioassays, all 20 males attempted to mate with dead females ($\chi^2 = 20$; d.f. = 1; $P < 0.001$) (N = 10) but did not respond to the same female after they had been washed in hexane, suggesting the evidence that females produce a cuticular contact pheromone which had been removed by the solvent, and that recognition was not cued solely by mechanoreception. However, 14 of 20 males attempted to male with dead-washed extract-treated females ($\chi^2 = 12.07$; d.f. = 1; $P < 0.001$), where immediately after antennal contact, the male mounted the female, rotating 180°, and clasping her pronotum or elytra with his forelegs and attempts to copulate. It therefore seems likely that a contact pheromone is involved in the precopulatory behaviour in *H. betulinus* males.

Our findings provide evidence that mate location in *H. betulinus* is mediated by a combination of a male-produced sex pheromone and a female-produced contact pheromone. The three male-produced components detected by GC are the main sex-pheromone candidates in the species. Research is now in progress aimed to identify and synthesize these chemical compounds.

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