Identification of Volatile Emissions from *Platypus mutatus* (=*sulcatus*) (Coleoptera: Platypodidae) and Their Behavioral Activity

PAOLA GONZALEZ AUDINO,[1] RAUL VILLAVERDE,[2] RENE ALFARO,[3] AND E. ZERBA

Centro de Investigaciones de Plagas e Insecticidas (CITEFA-CONICET), J-B. De LaSalle 4397 (B1603ALO), Buenos Aires, Argentina

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ABSTRACT We report here the identification and behavioral activity of volatile compounds emitted by male *Platypus mutatus* (=*sulcatus*) Chapuis while boring galleries in living poplar, *Populus deltoides* Marshall, trees. Headspace analysis using solid phase microextraction techniques showed the presence of 6-methyl-5-hepten-2-ol (sulcatol) and 6-methyl-5-hepten-2-one (sulcatone). Only one enantiomer of sulcatol, retusol, was found to be part of the volatile emission. Behavioral assays showed that females are more attracted than males to galleries with boring males inside. Both sulcatol and sulcatone elicited electroantennographic responses by female *P. mutatus*. Furthermore, behavioral bioassays showed that both sulcatol and sulcatone elicit behaviorally attractive responses by females. These results suggest that male *P. mutatus* releases a sex pheromone composed mainly of retusol and sulcatone.

KEY WORDS *Platypus*, sulcatol, sulcatone, pheromone, retusol

AMBROSIA BEETLES ARE AN IMPORTANT insect group affecting forest ecosystems by attacking mainly felled or weakened trees where their galleries and staining of wood cause damage to the lumber, reducing its market value. The name ambrosia is derived from the fungus on which they feed. *Platypus mutatus* (=*sulcatus*) Chapuis, 1865, is an ambrosia beetle (Platypodidae, Platypodinae, Platypodini) native to South America (Wood 1993), where it attacks only living standing trees, unlike most ambrosia beetles that attack cut wood or weakened trees. This insect drills into the trunks of live trees, boring large internal tunnels in the xylem, which weaken the stem, causing it to break under extreme stress. *P. mutatus* is a serious problem in commercial plantations of several broadleaf trees, but it is especially damaging to poplars (*Populus deltoides* Marshall) (Gimenez and Etiennot 2003). The dark-stained tunnels caused by the decay of the ambrosia mycelium (Bascialli et al. 1996) prevent the wood quality required for exporting.

Several ambrosia beetles species are known to respond to odors emanating from host trees. Secondary attraction and pheromone production have been intensively studied in ambrosia beetle species of the genera *Gnathotricus* and *Tripodendron*. Sulcatol (6-methyl-5-hepten-2-ol) was determined to be a component of the pheromones of *Gnathotricus sulcatus* (LeConte) (Byrne et al. 1974), *Gnathotricus sulcatus materiarius* Fitch (Fletchmann and Berisford 2003), and *Gnathotricus sulcatus retusus* (LeConte) (Borden et al. 1979).

In the genus *Platypus*, the male usually initiates the attack and gallery construction. *P. mutatus* do not mass attack their hosts. Attacks seem to occur at random and are distributed throughout the trees in a plantation (R. A., unpublished data). Pheromone production has been demonstrated for *Platypus apicalis* White, *Platypus gracilis* Broun, and *Platypus caviceps* Broun, although their chemical identities have not been studied (Milligan et al. 1988, Milligan and Ytsma 1988). Milligan and Ytsma (1988) indicate that *P. apicalis* and *P gracilis* exhibit mass-attack behavior mediated by aggregation pheromone, whereas *P. caviceps* attacks only sparsely and the males release a sex, rather than an aggregation pheromone. Sulcatol, 1-hexanol, and 3-methyl-1-butanol have been identified as volatile compounds produced by males of *P. flavicornis* (Renwick et al. 1977). The aims of this study were to determine the behavioral responses of males and females of *P. mutatus* to odors emitted by boring males in tunnels and to identify components of volatile emissions with potential pheromonal activity.

Materials and Methods

Biological Material. Insects were collected from a *Populus deltoides* plantation belonging to the company Papel Prensa S.A. located in Bragado, Province of Buenos Aires, Argentina. Emerging beetles were collected in plastic traps made with two tubes, 3 cm in diameter and 4 cm in length, connected in an L-

[1] Corresponding author, e-mail: pgonzalezaudino@citefa.gov.ar.
[2] Faculty of Forest Sciences, University of La Plata, Argentina.
[3] Canadian Forest Service, Pacific Forestry Centre, 506-W-Burnside Rd., Victoria, Canada V8N 4W7.

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shaped form so that when insects emerge from the gallery they walk horizontally through the first tube until they fall into the second, vertical tube. Traps were placed at the beginning of the season over *Platypus* entrance holes where the composition of the expelled sawdust suggested that emergence would occur soon. Emerged beetles were collected weekly, sexed, and placed in plastic boxes containing thick moistened sawdust and placed in an environmental chamber (Lab-Line Instruments, Melrose Park, IL) at 18 ± 0.5°C and 70–80% RH in the dark for further experimentation. This temperature is optimal for conservation of insects.

Synthetic Chemicals. Sulcatol, (±)-6-methyl-5-hepten-2-ol, and sulcatone, 6-methyl-5-hepten-2-one, were analytical grade (Sigma, St. Louis, MO). (+)-Sulcatol, 99% (chromatographic standard), was a gift from Pherotec, Vancouver, British Columbia, Canada.

Response of Beetles to Natural Galleries. To test the response of the beetles to volatiles emitted by boring males, an olfactometer was used. We placed two 10-cm-wide poplar billets inside an acrylic box. One billet contained a natural gallery induced 4 to 5 d before. The other billet had a blank artificial gallery, made by drilling a 4-mm-diameter hole. The billets were oriented with the entry holes facing upward. Both galleries were connected with a glass tube (3 cm in diameter). Each end of the tube had a 90° bend whose diameter was gradually reduced to fit into entrances of the galleries.

Beetles were introduced one at a time into the tube via an upper entrance, and the percentage of males or females walking or flying to the natural gallery over the total of responding (flying/walking beetles) was calculated. Each replicate was performed with a new pair of infested and uninfested billets.

Collection of Volatiles. Six living poplar trees were artificially infested with six virgin males and six virgin females. Individual beetles were introduced into a 4-mm drilled hole (Ytsma 1986) and in a plastic capsule.

Volatiles from an empty gallery were used as blank. So, to collect the host volatiles, on each tree a 4-mm-diameter hole was drilled close to the infested gallery but left uninfested. Volatiles were collected from individual gallery entrances where abundant and wet frass was present, 3–5 d after initial infestation. For volatile collection, the space over the gallery entrance was confined by inserting a glass tube (8 mm in diameter and 5 cm in length) and capped with a septum to the create headspace. The volatiles were collected using a solid phase microextraction holder (SPME) with a fiber of polydimethylsiloxane (Supelco, Bellfonte, PA).

Linked Gas Chromatography-Mass Spectrometry (GC-MS) Analyses. Analyses of SPME fibers were carried out by GC-MS on a Shimadzu QP 5050A instrument in electron impact mode. Samples were analyzed on a polar column (30 m by 0.32-μm film thickness, CP Wax 52CB Chrompack). Volatiles from the SPME fibers were desorbed at 250°C in the injector port for 1 min. The GC column was held at 50°C for 5 min. The temperature was programmed at 10°C/min to 220°C and held for 5 min. The carrier gas was helium with a head pressure of 14 kPa. The identities of compounds observed were confirmed by comparison of GC retention times and MS data with authentic compounds.

Electroantennography (EAG). The electroantennographic activity of sulcatol and sulcatone was tested following the method described previously (Fontán et al. 2002). Insects were immobilized on their dorsal surface in a notch made in a Plasticine block and restrained with a strip of polystyrene held in place with pins. The exposed antennae were firmly held on the surface of the Plasticine block by using U-shaped copper wires. Glass microelectrodes were made form borosilicate glass tubing (1.5 mm o.d., 0.84 mm i.d., Clark Electromedical Instruments, Reading, United Kingdom) and filled with Ringer solution (Fontán et al. 2002). Microelectrodes were held with micromanipulators (Leica, WILD Heerbrugg, Switzerland) and connected to a high-input impedance (10^{12} Ω AC/DC micro-amplifier model UN-06, Syntech, Hilversum, The Netherlands) via Ag/AgCl junctions. The recording electrode was inserted into the distal end of one antennal flagellum and the indifferent electrode into the basal scape of the same antenna. Amplified EAG responses were digitized, processed, and displayed on a PC by using EAG recording software (Syntech).

Pasteur pipettes containing test samples on filter paper strips (10 μg dissolved in dichloromethane) were positioned 1 cm above the mid-point of the EAG preparation. Test samples were exposed to the EAG preparation in a 2-s pulse of nitrogen (500 ml/min) through the pipette and over the preparation with a delay of at least 60 s between each replicate and sample. Each sample was tested at least twice with each EAG preparation, and controls (1 μl, dichloromethane) were run before and after each sample. Each series of samples was repeated in a randomized order with four different female *P. mutatus.* Mean EAG responses were divided by the mean of control signals taken before and after analysis

Behavioral Response of Females to Sulcatol and Sulcatone. The attractive effect of synthetic sulcatol and sulcatone to *P. mutatus* was tested using a Y-shaped glass olfactometer. Internal diameter of the olfactometer was 4 mm, similar to the diameter of the natural gallery, so that the insect could walk easily upwind in the airstream. A solution containing 10 μg of sulcatol or sulcatone in acetone was a deposited on a cotton wick and placed in one arm of the Y (source side), whereas in the other the control solvent was introduced (control side). Individual *P. mutatus* were introduced into the longitudinal arm of the Y from where responding insects walked toward the bifurcation, where they often stopped for a few seconds and then chose one of the branches and walked through it toward the source.

Statistical Analysis. The significance of the observed results of behavioral bioassays were analyzed using χ^2 tests.

Table 1. Number of male or female *P. mutatus* walking towards galleries containing a male

	Beetles walking towards gallery	
	Male	Female[a]
To natural gallery containing a male	14	39
To artificial gallery containing no beetle	13	10
Total of beetles responding	27	49

[a] Female *P. mutatus* were significantly more attracted than males to the natural gallery containing the boring male inside ($\chi^2 = 6.34$, df = 1, $P = 0.0119$).

Enantiomeric Determination of Sulcatol. Volatiles from induced galleries were collected with an SPME fiber as described above, and samples were analyzed on a Gamma-Dex 120 fused silica capillary column (Supelco), 30 m, 25 mm i.d., 0.25-μm film thickness with a temperature program starting at 50°C /1 min and 10°C /min to 220°C. Characterization of enantiomers was made by comparison and cochromatography of natural samples with a standard mixture of (+)- and (–)-sulcatol and with (+)-sulcatol, 99%.

Results

Behavioral Response of Beetles to Natural Galleries. Female *P. mutatus* were significantly more attracted than males to the natural gallery containing the boring male inside ($\chi^2 = 6.34$, $P = 0.0119$, df = 1; Table 1). Furthermore, in five of the eight replicates, 100% of females responded to the natural gallery, showing that they are more attracted to the volatile emissions from males than by the odors emitted by hosts (Table 1). Males showed no significant preference for galleries with males versus those without males.

As described previously, the fact that only females and not males are attracted to galleries containing males indicates that the potential pheromone is emitted by male *P. mutatus* and suggests it could be a sexual rather than an aggregation pheromone. This is supported by field observations that indicate that *P. mutatus* does not engage in mass attack behavior and that *Platypus* attack density per tree is sparse.

Volatile Collection and Analysis. Induction of attacks failed with females, as was observed previously in *G. materiarius* (Fletchmann and Berisford 2003). All the females failed to start boring in galleries. As described previously, males are the only sex that naturally initiates the galleries (Santoro 1962). So, volatiles were collected from active galleries containing males. The GC trace from the headspace samples from galleries containing males showed two main compounds (a and b) that were not present in the blank (control) system (Fig. 1). These were identified as 6-methyl-5-hepten-2-ol and 6-methyl-5-hepten-2-one.

EAG. Taking into account that females are attracted to males within galleries and that the volatiles emitted have been identified as sulcatol and sulcatone, it was of interest to test the EAG response produced by these compounds on female *P. mutatus.* The EAG responses elicited by sulcatol and sulcatone from female *P. mutatus* at a dose of 10 μg were significantly different from those of the solvent control. Mean EAG response to sulcatol was 0.31 ± 0.01 mV and to sulcatone 0.27 ± 0.01 mV. The behavioral bioassays showed that both sulcatol and sulcatone elicit a significant attraction by female *P. mutatus* (Table 2).

Enantiomeric Determination of Sulcatol. Chiral analysis of sulcatol by cocromatography with the authentic standard indicated that this compound was

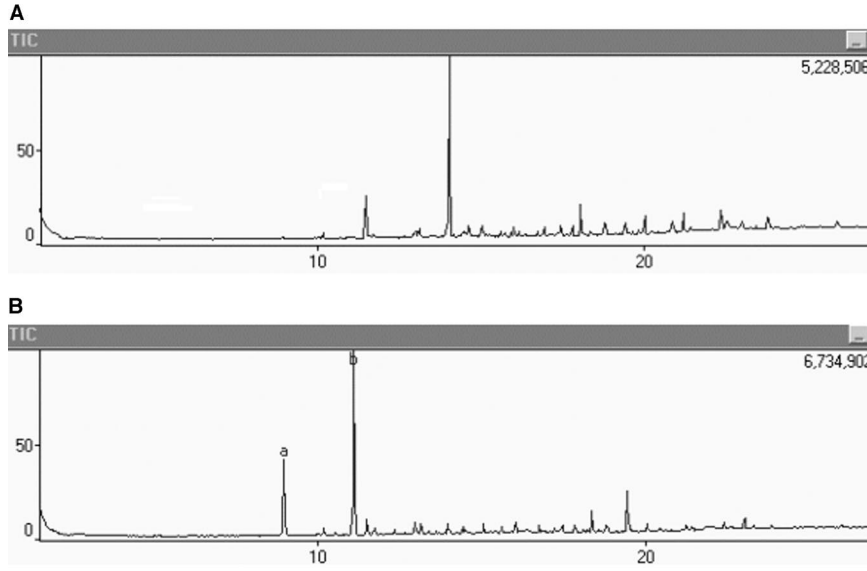

Fig. 1. (A) GC trace of an empty artificial gallery. (B) GC trace of a male containing natural gallery. Peaks marked as *a* and *b* are present only in male containing natural gallery.

Table 2. Number of female *P. mutatus* responding to sulcatol and sulcatone in a Y-tube laboratory olfactometer

Test chemical	No. of females walking to control	No. of females walking to test chemical
Sulcatol[a]	2	18
Sulcatone[a]	4	15

[a] χ^2 test on the number of insects responding to either sulcatol or sulcatone indicated that both chemicals elicited an equally significant attraction response on female *P. mutatus* compared with control. ($\chi^2 = 18.69$, df = 1, $P < 0.0001$).

present in the head space of males boring galleries as the (*S*)-(+)-isomer, also known as retusol.

Discussion

Attacks by *P. mutatus* are initiated by male and only males bore through the bark into the sapwood. Once boring the gallery, males release a volatile emission that in behavioral bioassays was attractive to females but not to males. That only females and not males are attracted to galleries containing males indicates that the potential pheromone is emitted by male *P. mutatus*, suggesting it could be a sexual rather than an aggregation pheromone. This is supported by *P. mutatus* not engaging in mass-attack behavior. Unlike bark beetles, where attack success depends on the phenomenon of mass attack to overcome tree resistance, *Platypus* attack density is sparse (<3 attacks per m^2 of bark).

The male-produced volatile composition of sulcatol and sulcatone may be indicative of the chemical composition of a *P. mutatus* pheromone. However, only one enantiomer of sulcatol, retusol, was found to be present in the volatile emission.

Both sulcatol and sulcatone elicited electroantennographic response by female *P. mutatus.* Furthermore, behavioral bioassays showed that both sulcatol and sulcatone elicited an attractive behavioral response by females.

These results suggest that males *P. mutatus* releases a sexual pheromone composed mainly by retusol and sulcatone. Sulcatol is known to be the pheromone or one of the pheromonal components for several ambrosia beetles. It is the single component of the pheromone of *G. sulcatus* (Byrne et al. 1974), *G. retusus* (Borden and Mc Lean 1979), and *G. materiarius* (Fletchmann and Berisford 2003), and it is part of the composition of the pheromones of many coleopteran pheromones (Tumlinson and Teal 1982). Sulcatone has not been previously reported as a component of ambrosia beetles pheromones.

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