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Anatomical site of pheromone accumulation and temporal pattern of pheromone emission in the ambrosia beetle *Megaplatypus mutatus*

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Abstract. *Megaplatypus mutatus* (Chapuis) is a native South American ambrosia beetle that attacks live hardwood trees (e.g. *Populus* spp.), causing important economic losses to commercial plantations. Male beetles release the main components of the sex pheromone, namely (+)-6-methyl-5-hepten-2-ol [(+)-sulcatol, or retusol] and 6-methyl-5-hepten-2-one (sulcatone), when colonizing suitable hosts. The hindgut is shown to be the anatomical site of pheromone accumulation within males, the enantiomeric composition of sulcatol in this tissue is 99%-(+) and sulcatol is first detectable in this tissue on days 1–2 after gallery initiation. Peak accumulation of sulcatol occurs on days 5–12 after gallery initiation. Trace quantities of sulcatone are also observed during the same period. Both pheromone components are present in male emissions from three host species (*Populus* × *canadensis*, *Populus alba* and *Casuarina stricta*) between days 2 and 12 after gallery initiation, although sulcatone is always present in low concentrations. The temporal patterns of sulcatol and sulcatone accumulation or storage in male *M. mutatus* correspond to the temporal patterns of emission.

Key words. *Megaplatypus mutatus,* pheromone emission, pheromone storage, *Platypus mutatus,* sulcatol, sulcatone, temporal pattern.

Introduction

Ambrosia beetles (Coleoptera: Platypodidae) are an important group of forest pests that colonize weakened or felled trees. Their name derives from the fungus that they inoculate when they penetrate the xylem of their host, and then later feed on as larvae. *Megaplatypus mutatus* (= *Platypus mutatus*) (Chapuis) is an ambrosia beetle native to South America (Wood & Bright, 1992; Wood, 1993) that only attacks standing live trees, mining deeply into the xylem through large tunnels, which are later colonized by the fungus (*Raffaelea santoroi*) that they transport. This activity weakens the structural integrity of the tree, causing severe stem-breakage and mortality in commercial plantations of poplar species such as *Populus deltoides* (Santoro, 1963; Achinelli *et al*., 2005; Alfaro *et al*., 2007). Gimenez & Etiennot (2003) reports that *M. mutatus* has an extensive host range, including hardwood trees in the genera *Populus*, *Quercus*, *Ulmus*, *Casuarina* and fruit trees. Similar to other ambrosia beetles, host colonization by *M. mutatus* is initiated when a male penetrates the bark and excavates a gallery of a few centimetres long. Using particles of boring dust (frass), the male beetle builds a crown-like arrangement surrounding the entrance to the gallery, from where it releases volatile emissions that attract females (Santoro, 1963; Milligan & Ytsma, 1988; Gonzalez Audino *et al*., 2005; Gatti Liguori *et al*., 2008).

A previous study reports on the collection of volatile emissions released from the nascent male gallery using a solid phase microextraction (SPME) fibre coated with a nonpolar, non-specific phase (Gonzalez Audino *et al*., 2005). After analysis by coupled gas chromatography-mass spectrometry (GC-MS), these samples show two main components: (+)-6-methyl-5-hepten-2-ol [(+)-sulcatol, or retusol] and the related ketone, 6-methyl-5-hepten-2-one (sulcatone). A behavioural bioassay with an olfactometer confirms that walking females move into

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air containing (+)-sulcatol or sulcatone. These results suggest that *M. mutatus* might emit a sex pheromone composed mainly of (+)-sulcatol and sulcatone (Gonzalez Audino *et al*., 2005). The activity of this blend is demonstrated in the field (Funes *et al*., 2009). Other platypodids (Renwick *et al*., 1977; Shore & McLean, 1983) and scolytids (Byrne *et al*., 1974; Borden & McLean, 1979; Flechtmann & Berisford, 2003) also respond to sulcatol but not to sulcatone.

An additional compound, 3-pentanol, is also found in a small percentage of samples of volatile emissions from male *M. mutatus* and females show a positive response to this compound (Gatti Liguori *et al*., 2008). However, its role in the context of the sex pheromone blend is not known and laboratory bioassay responses to the three-component blend are similar to the responses to the two-components blend.

In the present study, the anatomical site of accumulation of sulcatol and sulcatone is studied, as well as the temporal patterns of emission from males as they form galleries on three different host trees. The aim is to characterize the duration and quantitative range of pheromone emission so that the performance of controlled-release dispensers of the pheromone used in the field can be improved by modifying the release rates. To account for the broad host range of the ambrosia beetle, tree species available at the Buenos Aires Institute's plantation that are attacked by *M. mutatus* are used.

Materials and methods

Insects

Adult *M. mutatus* were collected shortly after their emergence (maximum 10 h) from infested *Populus* × *canadensis* Moench and *Quercus palustris* Münchh at the Centro de Investigaciones de Plagas e Insecticidas (34°33′S, 58°30′W), Buenos Aires, Argentina. Traps specifically designed for this beetle were used to avoid antagonistic interactions between males (Gatti Liguori *et al*., 2007). Only males were used in the experiments.

Chemicals

Racemic sulcatol [(±) 6-methyl-5-hepten-2-ol, 99% chemical purity), and sulcatone (6-methyl-5-hepten-2-one, 95% chemical purity) were analytical grade (Sigma-Aldrich Co., Saint Louis, Missouri). (+)-Sulcatol of 99% chemical and enantiomeric purity (chromatographic standard) was obtained from Contech Enterprises Inc., Delta British Columbia, Canada. Hexane (analytical grade) and 1-octanol (>98%) were purchased from Sigma-Aldrich Co.

Identification of site of accumulation of sulcatol and sulcatone

Male beetles that were boring galleries at an early stage of host colonization were collected, dissected in distilled water at 0 °C and homogenized; fat body, mesenteron, Malpighian tubules, genitalia and hindgut were each placed separately in a 50-μL hexane solution of 1-octanol 0.1 and 10 μg mL^{-1} (internal standard) for extraction. Tissue samples from five males were analysed, except for the small globular fat bodies where samples from three males were analysed.

Temporal pattern of the endogenous content and emission of sulcatol and sulcatone

To determine endogenous content of sulcatol and sulcatone, individual males were kept at −20 °C until dissection. Hindgut tissue was eviscerated in distilled water at 0 °C and homogenized in a hexane solution with 1-octanol as an internal standard. A total of 76 males were used to construct the curve (three insects per day).

To determine the temporal pattern of emission of sulcatol and sulcatone, live *P.* × *canadensis*, *Populus alba* (L.), and *Casuarina stricta* (L.) trees at least 10 years of age and with a diameter at breast height of over 20 cm were artificially infested with hundreds of recently emerged virgin males. Each insect was confined in a transparent plastic jar (60 × 30 mm) to ensure its interaction with the tree surface. Date and time of the initiation of gallery excavation by the male were recorded. *Populus* × *canadensis* experiments were performed from 24 November 2005 to 16 May 2006. *Populus alba* and *C. stricta* experiments were performed from 17 November 2006 to 4 May 2007. The volatile emissions of males in *P.* × *canadensis* were recorded over 52 days. Taking into consideration that, after 30 days, most of the pheromone values were below the detection limit, emissions from specimens on *P. alba* and *C. stricta* were only collected for the first 30 days.

At preset periods of time, males were extracted from the crown-like frass pile located outside their gallery with entomological forceps or a cork borer and placed in a 20-mL vial standard clear glass (Scientific Specialties Service, Inc., Baltimore, Maryland) with a teflon-coated cap (Teflon septum with glass reinforced polypropylene resin open cap). The number of males analysed was 126 for *P.* × *canadensis*, 123 for *P. alba* and 107 for *C. stricta*.

Chemical analysis

After verifying the physical integrity of each individual male, the volatiles from their headspace were collected at 30 °C for 30 min by using a SPME fibre covered with a Carbowax/Divinylbenzene (Supelco, Bellefonte, Pennsylvania) polar phase. This coating is specific for low molecular weight alcohols and ketones. Samples were immediately analysed by GC-MS (see below) and the areas corresponding to sulcatol and sulcatone were recorded. Mean estimates of pheromone quantity and the respective confidence intervals were calculated.

To evaluate the linearity of the semi-quantitative method, and considering that the SPME fibre has specificity for both alcohols and ketones, and also that sulcatol and sulcatone have similar molecular weights, sulcatol was chosen as the model

pheromone component for analysis. Different amounts of sulcatol (0.04–7 µg) were placed into empty vials, the SPME fibre was inserted and the system allowed to equilibrate at 30 ± 1 °C over 30 min. The fibre was then inserted into the injection port, the sample desorbed and a calibration curve was constructed based on the resulting areas of the sulcatol peaks (Magdic & Pawliszyn, 2002). The response was linear in the evaluated range (peak areas from 4.0×10^5 to 2.0×10^8) ($R^2 = 0.979$). The detection limit was 0.001 µg.

GC-MS analyses were performed with a Shimadzu QP-5050A spectrometer (Shimadzu Corp. Japan) in electron impact mode (70 eV), equipped with a HP-1 capillary column (Hewlett-Packard, Palo Alto, California), (50 m × 0.32 mm inner diameter × 0.25 µm film thickness). Samples were injected in splitless mode. Volatiles from the SPME fibres were desorbed in the injection port at 190 °C for 1.5 min. The GC column was kept at 60 °C for 1 min, after which the temperature was programmed to increase 3 °C min^{-1} up to 105 °C, and then 40 °C min^{-1} to 250 °C, where it was maintained for 5 min. The carrier gas was helium with a head pressure of 30 kPa.

For the enantiomeric determination of endogenous sulcatol, homogenates of individual hindgut were analysed on the same instrument as above but with a fused silica capillary column with a chiral stationary phase (Gamma-Dex 120; Supelco, Bellefonte, Pennsylvania) (30 m × 0.25 mm inner diameter × 0.25 µm film thickness). The column was maintained at 50 °C for 1 min after which the temperature was programmed to increase by 10 °C min^{-1} up to 220 °C. The carrier gas was helium with a head pressure of 30 kPa. Enantiomers were characterized by co-injection of natural samples with standard solutions of (±)-sulcatol and 99%-(+)-sulcatol.

Results

Identification of site of accumulation of sulcatol and sulcatone

Sulcatol and sulcatone were not detected in extracts of fat body, mesenteron, Malphigian tubules or genitalia (detection limit 0.04 µg mL^{-1}) but were detected in hindgut extracts. The content of sulcatol depended on the time that the male had been emitting pheromones from the gallery (see below) and sulcatone was always present in trace amounts. All sulcatol detected in the hindgut was the (+)-enantiomer (Fig. 1)

Temporal pattern of the endogenous content and emission of sulcatol and sulcatone

Sulcatol was observed in the hindgut after the first day of emergence, reaching a maximum concentration between days 5–12, and then decreasing until days 25–30 when it was no longer detected (Fig. 2). Sulcatone was present during the same period (days 2–12) but always in trace amounts. The highest concentrations detected were 21 µg $male^{-1}$ (sulcatol) and 0.6 µg $male^{-1}$ (sulcatone).

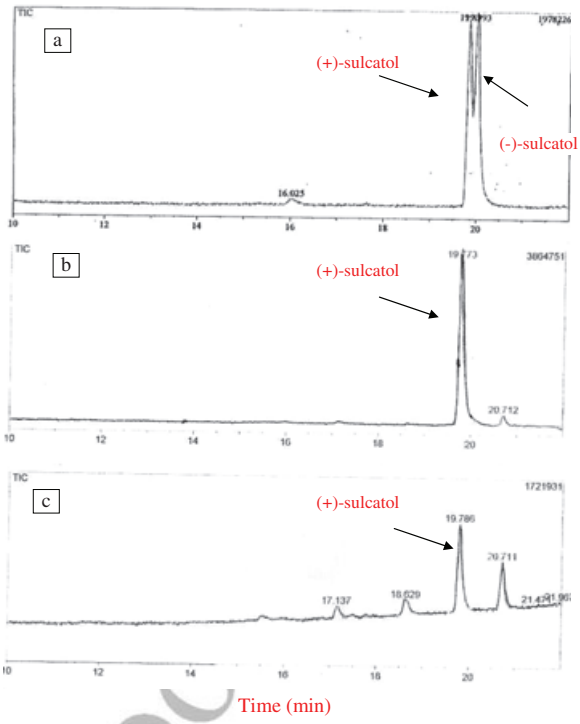

Fig. 1. (a) Determination of enantiomeric composition of sulcatol from the hindgut extract of *Megaplatypus mutatus* by chiral gas chromatography. (±)-Sulcatol, (b) (+)-sulcatol and (c) hindgut extract containing (+)-sulcatol.

Males that excavated galleries in *P.* × *canadensis* survived for a maximum of 52 days. The individuals that survived this long were not in a good physiological condition and did not appear capable of achieving courtship and reproduction in this host. The mean amounts of pheromone components emitted were 0.90 ± 0.14 µg $male^{-1}$ (sulcatol) and 0.18 ± 0.03 µg $male^{-1}$ (sulcatone). The mean ranges were 0.003–20 µg $male^{-1}$ (sulcatol) and 0.001–0.7 µg $male^{-1}$ (sulcatone) (Fig. 3).

There was a coincidence between storage time in the hindgut and the period of pheromone emission. However, because the collection methods differ, it was not possible to perform a quantitative comparison of the relative amounts of stored and emitted volatiles.

The amounts of sulcatol and sulcatone emitted by males induced to bore galleries in *P. alba* were 1.76 ± 0.21 and 1.64 ± 0.15 µg $male^{-1}$, respectively. The mean ranges were 0.007–12 µg $male^{-1}$ (sulcatol) and 0.009–7 µg $male^{-1}$ (sulcatone) (Fig. 4).

The amounts of sulcatol and sulcatone emitted by males in *C. stricta* were 1.20 ± 0.14 and 0.72 ± 0.08 µg $male^{-1}$, respectively. The mean ranges were 0.004–10 µg $male^{-1}$ (sulcatol) and 0.015–3.4 µg $male^{-1}$ (sulcatone) (Fig. 5).

Despite the large variability observed between insects, the GC traces of the emissions from all three host species showed that significant amounts of sulcatol are emitted between days 2 and 12. Nonetheless, trace amounts can be detected

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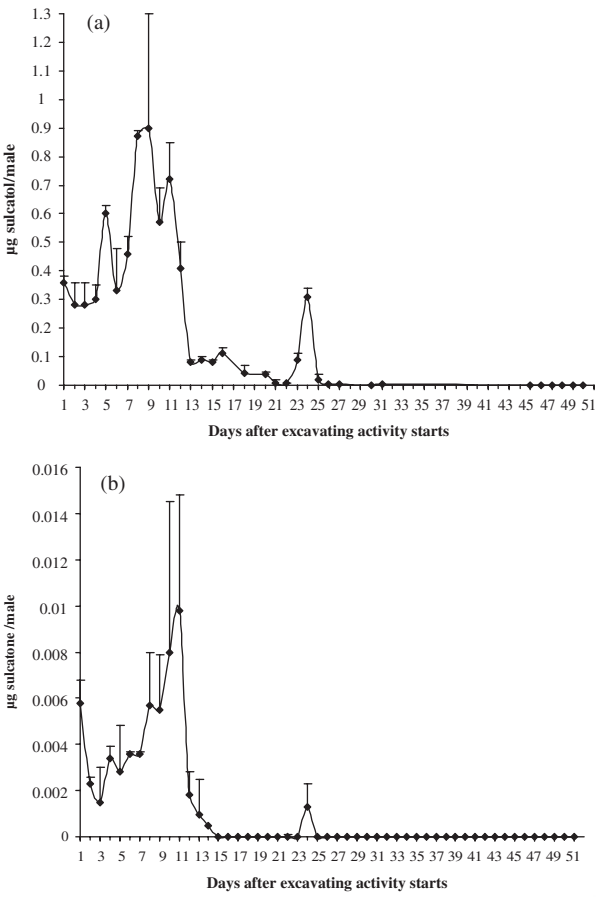

Fig. 2. (a) Daily temporal pattern of storage of sulcatol and (b) sulcatone in the hindgut of male *Megaplatypus mutatus* excavating galleries in its host *Populus* × *canadensis* ($n = 76$). Bars represent the standard error.

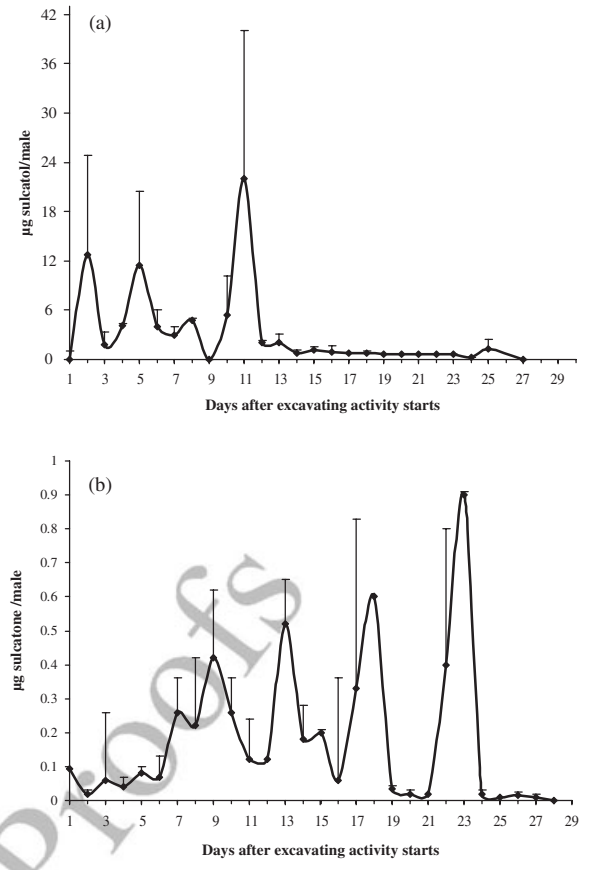

Fig. 3. (a) Daily volatile emissions of sulcatol and (b) sulcatone from male *Megaplatypus mutatus* excavating galleries in its host *Populus* × *canadensis* extracted by solid phase microextraction and analysed by gas chromatography-mass spectrometry ($n = 126$). Bars represent the standard error.

until approximately day 50 in the longest living example of *M. mutatus* in *P.* × *canadensis*.

Discussion

Analyses of the extracts of different *M. mutatus* body fractions show that sulcatol and sulcatone are only present in the hindgut. Quantities of these pheromone components are below the limit of detection in the other anatomical regions analysed. Hence, the hindgut appears to be the site of pheromone accumulation. Previous reports on bark and ambrosia beetles show that pheromones are generally localized to the alimentary canal, including the Malphigian tubules (Seybold & Vanderwel, 2003). Furthermore, sulcatol itself is reported as being isolated in hindgut extracts from the ambrosia beetle *Gnathotrichus sulcatus* (Byrne *et al.*, 1974). However, no information on the anatomical site of accumulation of pheromones is available for playtpodid ambrosia beetles.

Regarding the temporal pattern of the presence of sulcatol, sulcatol is not detected in the hindgut of recently emerged males and only reaches detectable amounts 1–2 days after gallery excavation. A delay in the presence of pheromone in abdominal tissue is also reported for *Ips* spp. pine bark beetles (Tillman *et al.*, 2004). This is attributed to the idea that a feeding-stimulated up-regulation of the pheromone biosynthetic enzymes may be necessary in the males. Adults of *M. mutatus* adults do not feed (Santoro, 1963; P. Gonzalez Audino, unpublished results) and so one explanation of this behaviour could be that males only start emitting pheromones once the gallery has reached a size of at least 1.5–2 body lengths because the female beetles must enter the gallery during courtship and expose their caudal region through the opening, after which mating takes place with the male outside the gallery (Santoro, 1963). Because this necessary length of gallery is only accomplished after approximately 48 h, males only begin pheromone release after this period of time.

The amounts of sulcatol in the hindgut reach their maximum between days 5 and 12 and then decrease. Sulcatone is present in the hindgut during the same period but only in trace amounts. The sulcatol detected in the hindgut is the (+)-enantiomer, which coincides with the sulcatol emitted in volatiles. This indicates that there is no enantiomeric biotransformation between stored and emitted sulcatol.

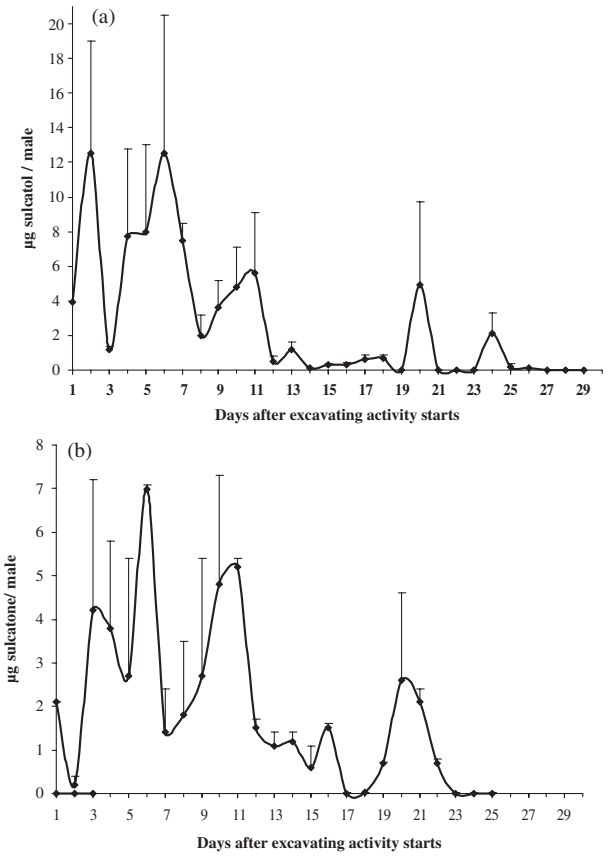

Fig. 4. (a) Daily volatile emissions of sulcatol and (b) sulcatone from male *Megaplatypus mutatus* excavating galleries in its host *Populus alba* ($n = 123$). Bars represent represent the standard error.

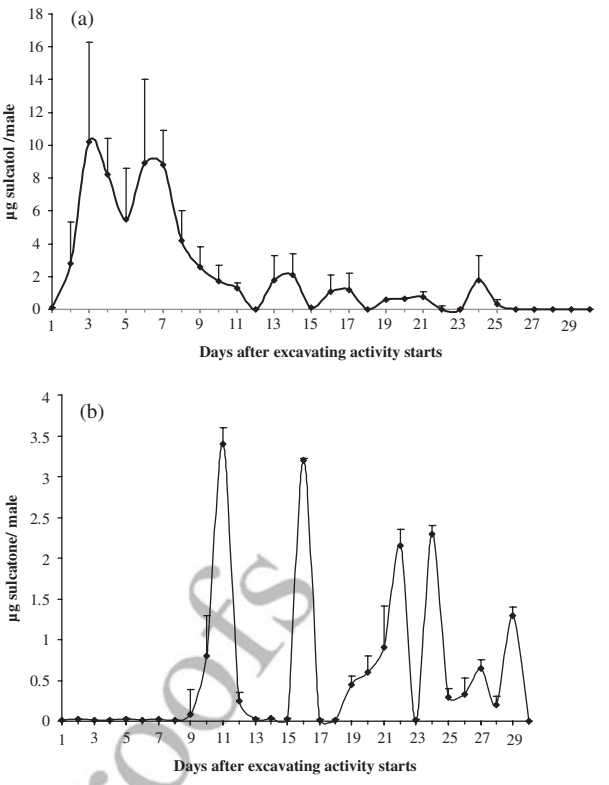

Fig. 5. (a) Daily volatile emissions of sulcatol and (b) sulcatone from male *Megaplatypus mutatus* excavating galleries in in its host *Casuarina stricta* ($n = 107$). Bars represent the standard error.

Because adult *M. mutatus* do not feed on host phloem and/or xylem, the initiation of sulcatol synthesis is not induced by feeding on host precursors but appears to be influenced by the emergence from host or pupa or by dispersal flight. Sulcatol is already reported to be a beetle-produced compound in another ambrosia beetle *Gnathotrichus sulcatus* because these beetles do not feed at all until the arrival of the females (Byrne *et al*., 1974).

The temporal pattern of volatile emissions from the three host species studied is in the same order and most sulcatol is emitted between days 2 and 12. This similar pattern of sulcatol emission from male *M. mutatus* in all hosts is expected because it is most likely a beetle-produced compound (Byrne *et al*., 1974). On the other hand, although sulcatone is always present in lower quantities, the temporal patterns of emission are not constant in the three hosts. However, these differences in sulcatone emission could also be a result of an experimental measuring error in the sulcatone determinations owing to the minimal quantities analysed.

Great variability is observed between the amounts of pheromone emitted among insects. This may be attributed to the samples being all collected during the day, whereas *M. mutatus* are particularly active at sunrise (Santoro, 1963), and so the highest emission of pheromones would be expected early in the morning, after which it would decrease throughout the day.

Significant amounts of sulcatol, but not sulcatone, are stored in the hindgut. However, both sulcatol and sulcatone are detected in the volatile emissions. Sulcatol might be transformed to sulcatone immediately before (inside the digestive system) or as it is expelled from the digestive system (externally). Sulcatol may be a reserve compound that is transformed to sulcatone until the supply is completely depleted. If this is the case, the source of sulcatol biosynthesis is not renewable and so males should achieve maximum efficiency when attracting females. This may explain why the rate sulcatol/sulcatone emitted in volatiles is not constant but, as time passes and the reserves of sulcatol decrease, the sulcatol/sulcatone ratio emitted decreases for the three hosts studied (Fig. 6)

Regarding the external transformation of sulcatol to sulcatone, it is reported that sulcatone is produced by microorganisms associated with scolytid species. The southern pine beetle *Dendroctonus frontalis* is closely associated with two forms of *Ophiostoma*, both of which produce sulcatone and a number of monoterpenes (Brand & Barras, 1977). In the present study, however, the fungus associated with *M. mutatus* (*R. santoroi*) only proliferates once the males are coupled with females and so it is unlikely that the fungus is responsible for the conversion of sulcatol to sulcatone. Nonetheless, the hypothesis that the symbiotic fungus, or other nonspecific microorganisms, may be involved in pheromone biosynthesis in *M. mutatus* cannot

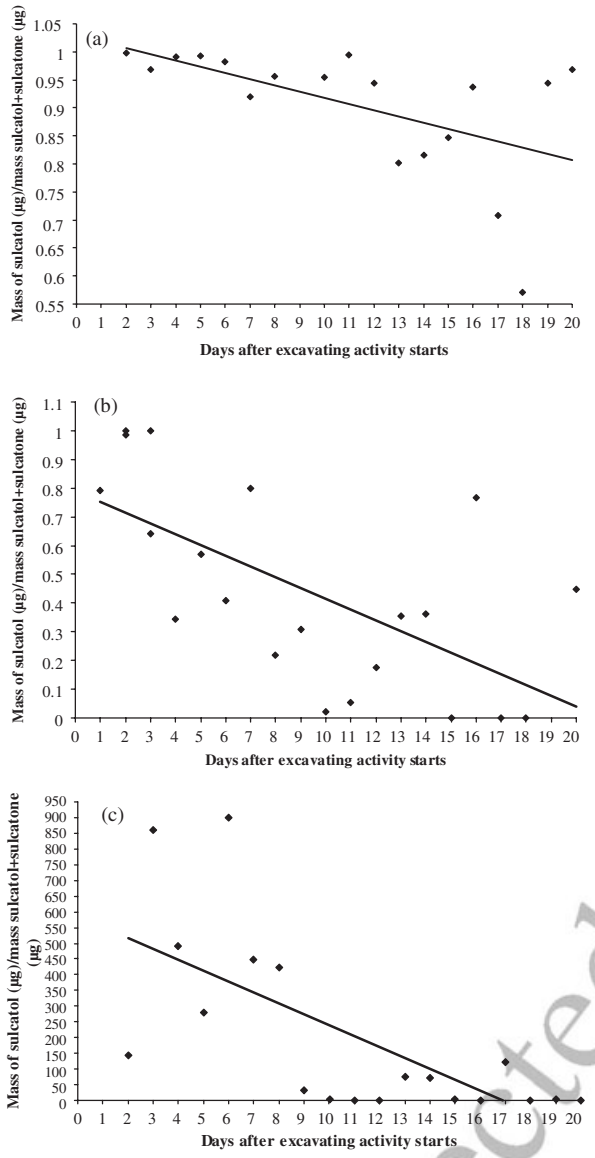

Fig. 6. Daily rates of mass of sulcatol/mass of (sulcatol + sulcatone) emitted by males *Megaplatypus mutatus* excavating galleries in different hosts. (a) *Populus* × *canadensis*, (b) *Populus alba* and (c) *Casuarina stricta.*

be disregarded. Current work involves the inhibition of this process using antibiotics (Byers & Wood, 1981). By contrast, sulcatone might be produced and released so quickly that none remains stored in the beetle (Gries *et al*., 1988) or it could be produced or concentrated somewhere else other than the hindgut. Current work is also aiming to determine the mechanism of conversion of sulcatol to sulcatone by *in vivo* and *in vitro* inhibition of the possible enzymes involved in this process. Whether the associated fungus has any role in the sulcatol-sulcatone conversion is also being studied.

In conclusion, the hindgut comprises the storage site of the pheromonal components (+)-sulcatol and sulcatone in *M. mutatus*. Moreover, characterization of the contents in the hindgut and volatile emissions show a qualitative correlation between the pheromone storage period and their emission. A knowledge of the storage and emission of (+)-sulcatol and sulcatone is useful for understanding the temporal pattern of emergence of the *M. mutatus* population. The pheromone emission period takes place from days 2–12 after initiating the attack on the host. This period needs to be confirmed by field observations of female attraction to males as well as the colonization behaviour of *M. mutatus*.

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AQ3. 'It is reported that sulcatone is produced by microorganisms associated with scolytid species'. Please clarify the specific reference being referred to here.

AQ4. 'Current work is also aiming to determine…'. Please clarify who is carrying out this work (i.e. the present authors or someone else?).