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Role of solid-phase microextraction in the identification of highly volatile pheromones of two Rhinoceros beetles *Scapanes australis* and *Strategus aloeus* (Coleoptera, Scarabaeidae, Dynastinae)

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Abstract

Solid-phase microextraction (SPME) samplings from live insects or natural secretion allowed one to identify the aggregation pheromones of the pest beetles *Scapanes australis* and *Strategus aloeus* by efficient and rapid isolation of their highly volatile ($72<M_r<116$) components. *S. australis* male pheromone was identified as a 84:12:4 (w/w) mixture of 2-butanol [67:33 (*R*)-(−):(*S*)-(+) ratio], 3-hydroxy-2-butanone and 2,3-butanediol [43:17:40 (*R*,*R*)-(−):(*S*,*S*)-(+):*meso* ratio], and *S. aloeus* pheromone as a 95.5:4.0:0.5 (w/w) mixture of 2-butanone, 3-pentanone and *sec*.-butyl acetate by GC–MS using conventional and chiral capillary columns. This is the first report of Scarabaeidae pheromones based on such small and common molecules. © 2000 Elsevier Science B.V. All rights reserved.

Keywords: Scapanes australis; *Strategus aloeus*; Extraction methods; Headspace analysis; Solid-phase microextraction; Pheromones; Volatile organic compounds

1. Introduction

A classical method of isolating insect pheromones consists of trapping airborne volatile organic compounds (VOCs) onto an absorbent matrix (activated charcoal; organic polymers) and eluting the trapped chemicals with a solvent [1]. Thermal desorption of the trap was possible but required costly desorption systems coupled to the analytical devices until recently [2]. SPME (solid-phase microextraction) has facilitated both the rapid isolation and analysis of airborne pheromones mainly by reducing the time of sampling and keeping a high sensitivity that allows experiments on a few insects [3–5]. As SPME is a solvent-free technique (thermal desorption in conventional gas chromatograph injectors), it offers the possibility to analyse highly volatile and/or trace compounds that cannot be easily disclosed by gas chromatography (GC) when organic solvents are used (co-elution with the solvent or loss during concentration). Finally SPME allows one to sample chemicals from living organisms with few disturbances in (semi-) natural environments, necessary to the performance of peculiar behaviours associated with the chemical release.

Scapanes australis Bsdv. and *Strategus aloeus* (L.) are 5-cm long Rhinoceros beetles. They are

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major pests on young coconut and oil palms, respectively, in Papua New Guinea (PNG) and Latin America [6,7]. The discovery of pheromones in two related *Oryctes* species [8–10] allowed the development of mass trapping using synthetic pheromone and improved the pest control. We investigated the existence of pheromones in *S. australis* and *S. aloeus* with the same objective. Trappings of airborne VOCs from *S. australis* eluted by organic solvents [11] and coupled to extensive bio-screenings and GC analyses failed in evidencing pheromone bioactivity and insect specific compounds during several months [12]. Further behaviour observations revealed the existence of nocturnal male calling behaviours in both species, which were correlated to the attraction of both sexes to the males in the field. The calling behaviours were associated to the emission of odours perceptible to humans and a liquid secretion in *S. australis* [13,14]. We report here the use of SPME for the isolation and identification of the pheromones of these two Rhinoceros beetles.

2. Experimental

2.1. *Scapanes australis*

2.1.1. *Isolation of natural pheromone*

Males were individually settled in artificial galleries prepared in sugarcane stalk pieces so as they adopted the nocturnal calling behaviour in a room at 28°C. Droplets of male secretion were collected by capillarity from the tip of the abdomen of calling individuals using 10-μl glass micropipettes (first to third hours of scotophase; 12:12 L:D regime). The micropipettes filled with secretion were immediately stored in a 2-ml glass vial kept in a methanol–water ice bath (−12°C) during the sampling period. They were further stored at −40°C. Liquid faeces from males and females emitted on aluminium sheets were collected and handled using the same procedure (fourth to the first hour prior to the scotophase). Direct injections of 0.1 μl pure male secretion were made for GC and GC–mass spectrometry (GC–MS) analyses (n=5). After some rapid preliminary assays varying the secretion amounts (1–5 μl) and the equilibrium (30–60 min) and sampling (5–60 min) times, the following protocol was retained: 1 μl of male secretion (n=11) or 1 μl faeces (male: n=7 and female: n=6) was deposited in a 2-ml glass vial fitted with a PTFE-lined septum for SPME sampling. The sample was left for 30 min at 21–23°C for droplet vaporisation and headspace equilibrium in the vial. VOCs emitted by the droplets were then sampled by exposing a SPME 65-μm polydimethylsiloxane–divinylbenzene (PDMS–DVB) coated fibre (Supelco, Bellefonte, PA, USA) in the glass vial for 20 min. The samples were desorbed for 5 min in the chromatograph injectors for analyses.

2.1.2. *SPME use for rapid design of synthetic pheromone mixtures*

To proceed with field testing of a synthetic *S. australis* pheromone, mixtures of (±)-2-butanol, 3-hydroxy-2-butanone and (±)-2,3-butanediol were prepared (see Table 3 for composition). A 1-ml volume of pure mixture was placed in a 30-ml open glass vial. Headspace pheromone was sampled after 5 min equilibration by exposing a PDMS–DVB fibre for 15 s 1 cm above the vial neck (21–23°C). The composition of the SPME-sampled pheromone was described based on the ratios of the corresponding GC peak areas and compared to that of the natural pheromone. The synthetic mixture was then empirically modified step-by-step to fit to the SPME–GC image of the natural pheromone. The pheromone was analysed as described in Section 2.3.3.

2.1.3. *Bioassay for pheromone activity*

A 90:5:5 (v/v) mixture of (±)-2-butanol, 3-hydroxy-2-butanone and (±)-2,3-butanediol (see Section 2.4) was tested for biological activity at the CCRI Tavilo plantation (East New Britain, PNG; 25 August–12 September 1998). The traps consisted of modified 18-l plastic buckets with an inner box containing sugarcane (SC), and one live male in controls. Soapy water poured at the bottom of the bucket retained the lured beetles. The pheromone was formulated in open 25-ml polyethylene (PE) flasks (Kartell, Noviglio, Italy) hung in the bucket traps. The flasks were opened and placed in the field only during the period of *S. australis* activity: 17:00 to 20:30 [13], and kept closed in freezer in the meantime. The trial comprised four treatments: one live male+SC (control), SC only, synthetic mixture only and synthetic mixture+SC, disposed in eight

complete blocks. It consisted of three replicates over six days. The traps were spaced by 50 m along one row in each block. Two blocks were 100-m distant. The catches were recorded daily when the synthetic mixture was removed from the traps. SC was renewed and the treatments were re-randomised weekly. The dispensers were weighed prior to and at the end of each replicate to measure the release rate of the mixture. Total catches per trap during one replicate were processed by a two-way (F1: bait; F2: weekly replicate) analysis of variance (ANOVA) on ln $(x+1)$ transformed data. The means were compared by Newman–Keuls tests at 5% [15].

2.2. *Strategus aloeus*

2.2.1. *Isolation of natural pheromone*

Insects were individually placed in plastic boxes filled with 30-cm soil and installed in a room at 28°C so as they dig a gallery and males adopted the calling behaviour at the gallery entrance. The pheromone was SPME-sampled directly at the entrance of the gallery during the male calling period (eighth to the eleventh hour of scotophase; 12:12 L:D regime) by exposing a 75-μm Carboxen–polydimethylsiloxane coated (CAR–PDMS) SPME fibre at ca. 1 cm from the gallery entrance (insect abdomen) for 5 min ($n=4$). Control samplings (male or female, deep inside the gallery; $n=5$) were done between the third and fifth hours of scotophase (out of the calling period).

2.2.2. *Validation of SPME conditions: fibre choice and sampling time*

To validate the choice of the SPME fibre and optimise the conditions of further studies, we compared the amounts of pheromone compounds collected by a CAR–PDMS fibre and a PDMS–DVB fibre according to the sampling time. The work was achieved using a 10% aqueous solution of a reference synthetic mixture of 2-butanone, 3-pentanone and (±)-*sec*.-butyl acetate (see Section 2.4) in a 99.8:0.1:0.1 (v/v) ratio, the vapours of which roughly mimicked the natural pheromone. The vapour emitted from 1 μl of the aqueous solution in 16-ml glass vials fitted with PTFE-lined septa was sampled simultaneously by the two fibres from the same vial for 1, 2, 5, 10 and 20 min at 21°C, after a 30-min vaporisation and equilibration at 28°C (five replicates).

2.2.3. *Bioassay for pheromone activity*

The response of the adults ($n=16$: 9♂, 7♀) to 20 μg of a 99.8:0.1:0.1 (v/v) mixture of 2-butanone, 3-pentanone and (±)-*sec*.-butyl acetate (10 μg/μl aqueous solution) was studied in a large four-way olfactometer (Laucoin, Saint-Cyr-L'Ecole, France) adapted from Vet et al. [16]. The system consists of a walking chamber with four contiguous dynamic air fields that can be independently odorised. The test insect, initially placed at the centre of the chamber, can move freely from one field to another. A 2-μl volume of pheromone solution was deposited onto a filter paper, placed in a glass vial connected to one way of the olfactometer. A 500 ml/min flow of moistened and purified air passed through each way. Insects were tested individually during the scotophase at 27°C for 10 min. The time spent in each way was recorded using the EVEN software [17]. The times spent in the three control ways were cumulated and compared to the time spent in the pheromone odorised way by a binomial test (null hypothesis of spending 25% time in the pheromone odorised way and 75% time in the control ways).

2.3. *Analytical procedures*

2.3.1. *Natural pheromone quantification*

Quantification of the pheromone compounds were realised after flame ionisation detection (FID) response calibrations from titered solutions of the six reference pheromone components (see Section 2.4). The calibration factors of the SPME fibres used for the pheromone compounds were calculated to estimate the w/w ratio of the pheromone components in the headspaces. Calibration factors (K), which reflect the affinity of the fibres for the compounds were calculated with the formula from Bartelt [18] and using a similar protocol. Affinities of a PDMS–DVB fibre for (±)-2-butanol, 3-hydroxy-2-butanone and (±)-2,3-butanediol and of a CAR–PDMS fibre for 2-butanone, 3-pentanone and (±)-*sec*.-butyl acetate were calculated. A 1-μl volume of four aqueous solutions of the six pheromone compounds (50, 100, 500 and 1000 ng doses) was left to vaporise for 30 min in 16-ml vials fitted with PTFE-lined septa

(21–23°C). The headspaces were then sampled for 20 min (28°C). A single measure was done per dose. If the ''fibre-trapped amounts vs. final headspace concentration'' adjustments (r^2) were lower than 0.95 a second set of measures was recorded.

2.3.2. GC–MS analyses

Spectra were recorded on a GC–MS R10-10C Nermag (Quad Service, Poissy, France) quadrupole instrument in electron impact mode (EI) at 70 eV. Molecular masses were confirmed by chemical ionisation at 92.5 eV using ammonia as reagent gas (CI–NH_3) with a $1.33322 \cdot 10^{-2}$ Pa ion source pressure. Samples were introduced via a Varian 3300 gas chromatograph equipped with a septum programmable injector (SPI) maintained at 240°C and two types of columns: an apolar 30 m×0.32 mm I.D., 0.5 μm d_f, Rtx-5MS (Restek, Bellefonte, PA, USA) operated under conditions **I**: 1 min at 50°C, 50°C to 240°C at 10°C/min; and a chiral 30 m×0.32 mm I.D., 0.25 μm d_f, Rt-βDEXse (Restek), operated under conditions **II**: 1 min at 50°C, 50°C to 54°C at 1°C/min and 54°C to 140°C at 4°C/min or **III**: 1 min at 15°C (cryogenic oven cooling), 15°C to 45°C at 10°C/min, 1 min at 45°C, and 45°C to 140°C at 2°C/min with helium as carrier gas (0.7 bar inlet pressure) under all three conditions. The chirality determinations of the compounds from *S. australis* were achieved with SPME sampling for 2-butanol, and by direct injection of the male secretion for 3-hydroxy-2-butanone and 2,3-butanediol (mixed secretions from five individuals).

2.3.3. GC analyses

Analyses of natural and synthetic compounds were conducted on a Varian 3400 CX gas chromatograph equipped with a SPI (240°C) and a FID system (240°C). They were realised on both polar and apolar columns using helium at 0.7 bar inlet pressure as carrier gas.

The best separations of *S. australis* compounds were typically obtained from the apolar column used in GC–MS and a polar 30 m×0.32 mm I.D., 0.25 μm d_f, DB-WAX (J&W Scientific, Folsom, CA, USA) column, both operated under conditions **IV**: 1 min at 35°C, 35°C to 80°C at 20°C/min, 1 min at 80°C, 80°C to 220°C at 5°C/min. Samples were desorbed from the SPME fibres for 5 min.

The best separations of *S. aloeus* compounds were obtained from the apolar column operated under conditions **V**: 1 min at 15°C (oven cryogenic cooling), 15°C to 40°C at 10°C/min, 1 min at 40°C, and 40°C to 80°C at 5°C/min, 1 min at 80°C, 80°C to 120°C at 10°C/min; and the polar column operated as for *S. australis*. Samples were desorbed from the SPME fibres for 1 min.

2.4. Reference compounds

The following reference chemicals were used for compound identification, SPME calibration and bioassays: (±)-2-butanol [=(±)-*sec*.-butanol] (>99% GC purity; Aldrich, Gillingham, UK: source 1), (*R*)-(−)-2-butanol (>99% GC purity; enantiomeric excess (e.e.) 99% GC; Aldrich, Milwaukee, WI, USA: source 2), (*S*)-(+)-2-butanol (>99% GC; e.e. 99% GC; Fluka, Buchs, Switzerland: source 3), 3-hydroxy-2-butanone (>97% GC; mixture of dimer and monomer; source 3), (±)-2,3-butanediol (>98% GC; mixture of (2*R**,3*R**) and *meso* forms; source 1), (2*R*,3*R*)-(−)-2,3-butanediol (>99% GC purity; e.e. 98% GLC; source 1), (2*S*,3*S*)-(+)-2,3-butanediol (>99% GC purity; e.e. 99% GLC; source 2), 2-butanone (>99.5% GC; Janssen, Belgium), 3-pentanone (>98% GC; source 1), (±)-*sec*.-butyl acetate [=(±)-1-methylpropyl acetate] (>99% GC; source 2). Titered solutions of these were prepared with water, acetone or methylene chloride (>99.9% GC; SDS, Pépin, France) as required.

3. Results

3.1. Scapanes australis pheromone

3.1.1. Chemical identification

GC (polar and apolar columns) and GC–MS (apolar column) analyses of the SPME samples revealed the existence of one major peak and three minor ones characteristic of the male pheromone secretion (Fig. 1a). Female faeces did not contain those peaks, or traces at the corresponding retention time (t_R) values in some cases (Fig. 1b); male faeces collected during day time contained 10-times lower amounts of the major but none, or occasional traces, of the minor peaks (Table 1). EI mass spectra

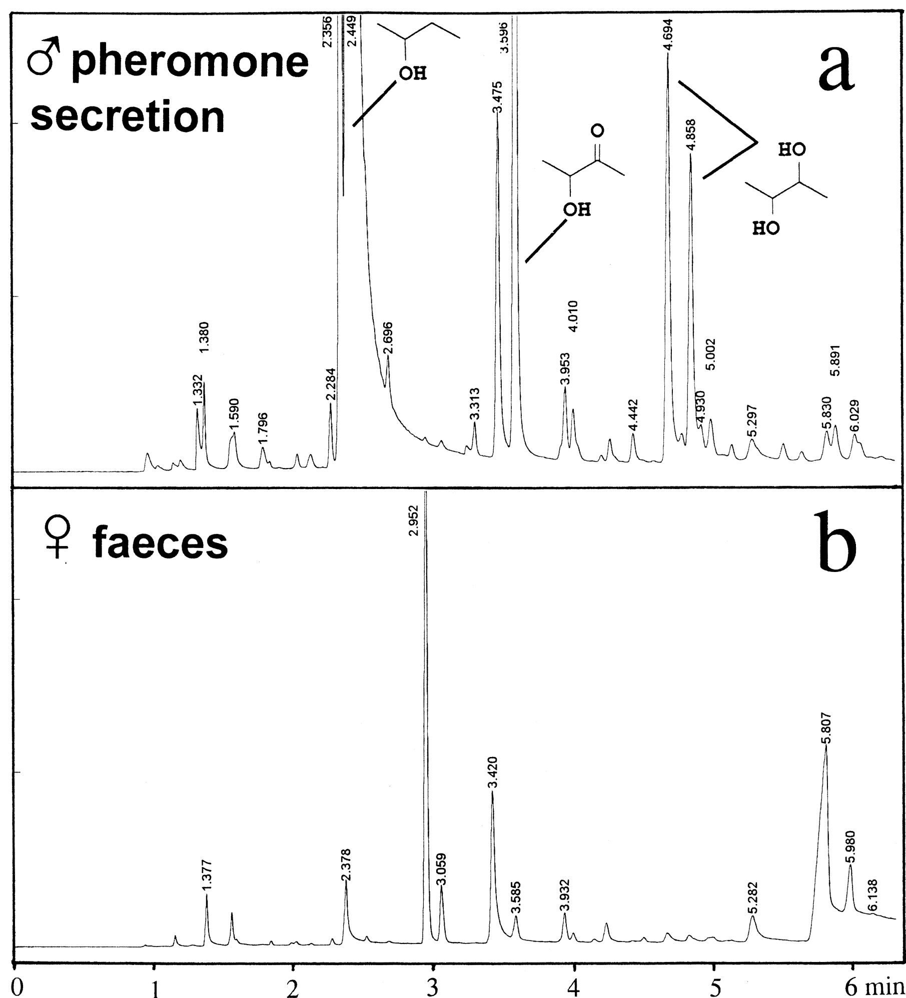

Fig. 1. Chromatograms of PDMS–DVB SPME-sampled volatiles from 1 μl male *Scapanes australis* pheromone secretion (a) and 1 μl female faeces (b) evidencing the three components of the male pheromone (sample origin and sampling conditions in Section 2.1.1; GC analyses on the apolar column under conditions **IV** (see Section 2.3.3).

enabled us to tentatively identify these compounds as 2-butanol (major peak), 3-hydroxy-2-butanone and 2,3-butanediol. Molecular masses were confirmed by CI–NH_3 at 74, 88 and 90 u, respectively. Reference chemicals analysed in the same conditions gave the same t_R values and spectra identical to the natural products.

A 1-μl volume of male secretion treated and sampled as described above emitted a total 1.79±0.17 (standard error, S.E.) μg pheromone compounds in a 84:12:4 (w/w, in headspace) ratio of 2-butanol, 3-hydroxy-2-butanone and 2,3-butanediol, respectively, on average. From the direct GC injections, 0.1 μl of pure secretion contained 0.65±0.19 (S.E.) μg pheromone in a 56:21:23 (w/w) ratio on average. The composition of the pheromone sampled in headspace appeared stable (Table 1). The amounts of 3-hydroxy-2-butanone were difficult to measure from the pure material because this compound eluted as an asymmetric broad peak in several cases on the apolar column. This problem did not occur on the polar column. The *meso* form of 2,3-butanediol accounted for 40% in the natural secretion.

Table 1
Qualitative and quantitative characteristics of *Scapanes australis* male pheromone by GC and GC–MS after SPME sampling and direct injection (mass or ratio values correspond to mean±standard error)

Molecule	2-Butanol	3-Hydroxy-2-butanone	2,3-Butanediol
M_r	74	88	90
K constant PDMS–DVB (ml)[a]	4.40	5.07	0.86
Male pheromone secretion			
Direct injection (ng in 0.1 μl) (n=5)	413±165	116±26	120±40
% (w/w) in secretion	56±7	21±4	23±5
Headspace amounts (ng from 1 μl)[b] (n=11)	1543±191	183±32	63±10
% (w/w) in headspace	84±3	12±2	4±1
Male faeces			
Headspace amounts (ng from 1 μl)[b] (n=7)	134±36	tr[c]	nd[c]

[a] Calculated at 21–23°C under the conditions described in Section 2.1.2.
[b] Total amounts (adsorbed on the PDMS–DVB SPME fibre plus present in the 2-ml headspace at the end of the sampling) from 1 μl pheromone secretion; detailed conditions in Section 2.1.1.
[c] nd=Not detected; tr=traces.

Reference (±)-2-butanol gave two peaks at t_R values 6.3 [(2*R*)-(−)] and [(2*S*)-(+)] 6.6 min with a 50:50 ratio with the chiral phase (conditions **II**). The order of elution of the isomers was determined by comparison with the reference enantiomers. Natural secretion gave two peaks at the same t_R values and with a 67:33 ratio of the (2*R*) and (2*S*) enantiomers, respectively (Table 2). Reference 2,3-butanediol gave three peaks at t_R values: 13.7, 14.2 and 14.7 min in a 17:17:66 ratio (conditions **III**). The (2*R*,3*R*)-(−)- and (2*S*,3*S*)-(+)-2,3-butanediol eluted at 13.6 and 14.1 min, respectively. The third peak with t_R=14.7 min was attributed to the *meso* form. Natural male pheromone secretion gave three peaks at the same t_R values with a 43:17:40 ratio (Table 2). The reference 3-hydroxy-2-butanone gave two peaks at t_R values 7.2 and 8.9 min with a 50:50 ratio (conditions **III**). The second one at t_R 9.0 min was the major one (94%) in the natural secretion (Table 2). Attribution of those peaks with identical EI and CI–NH_3 mass spectra to a specific form was not investigated.

3.1.2. SPME use for rapid design of synthetic pheromone mixtures

Though carried out in different conditions than for natural pheromone, SPME sampling allowed a rapid optimisation of synthetic mixtures mimicking the

Table 2
Retention times and proportions of the isomers of synthetic and natural components of *Scapanes australis* male pheromone by GC–MS on chiral capillary column

Molecules	2-Butanol		3-Hydroxy-2-butanone		2,3-Butanediol		
Isomers or "forms":	(*R*)-(−)	(*S*)-(+)	F1[a]	F2[a]	(2*R*,3*R*)-(−)	(2*S*,3*S*)-(+)	*meso*
Reference molecules							
t_R (min)	6.3[b]	6.6[b]	7.2	8.9	13.7	14.2	14.7
Isomer ratio (%)	50[b]	50[b]	50	50	17	17	66
Natural male pheromone secretion							
t_R (min)	6.3[b]	6.6[b]	7.3	9.0	13.7	14.2	14.7
Isomer ratio (%)	67[b]	33[b]	6	94	43	17	40

[a] F1 and F2 correspond to two undetermined forms of 3-hydroxy-2-butanone, the EI and CI–NH_3 mass spectra of which were identical.
[b] Retention times (t_R) were recorded from GC–MS analyses on chiral column operated under conditions **II** (labelled [b]) or **III** (no label); see Section 2.3.2.

Table 3
SPME monitoring of the headspace composition from three-component synthetic mixtures to reproduce *Scapanes australis* male pheromone: correspondences of v/v ratios in liquid mixtures versus GC peak area ratios in headspaces from SPME samplings

Chemical source	Ratios (% total)	2-Butanol	3-Hydroxy-2-butanone	2,3-Butanediol
Natural pheromone (n=11)	Headspace: FID[a]	86.0±2.5	12.0±2.5	2.0±0.0
Synthetic mixture 1	Liquid: v/v[b]	50	25	25
(n=3)	Headspace: FID	45.5±2.0	43.5±3.0	11.0±2.5
Synthetic mixture 2	Liquid: v/v	87	8	5
(n=3)	Headspace: FID	76.5±1.0	22.5±1.0	1.0±0.0
Synthetic mixture 3	Liquid: v/v	91	4	5
(n=4)	Headspace: FID	87.0±1.0	11.0±1.0	2.0±0.5

[a] Area proportions of the GC peaks (apolar column, conditions **IV**; see Section 2.3.3) in the headspace sampled by PDMS–DVB SPME (see Section 2.1.1 for natural pheromone and Section 2.1.2 for synthetic mixtures). Values correspond to means±standard errors.
[b] Volume/volume ratio in the synthetic liquid mixture (fixed). Crystallised 3-hydroxy-2-butanone was heated to melt.

natural pheromone of *S. australis* without determining K constants or FID response calibrations. The ratios (GC peak areas) of the *S. australis* pheromone components in headspace followed the ratios in the liquid mixtures (Table 3). A 90:5:5 mixture of (±)-2-butanol, 3-hydroxy-2-butanone and 2,3-butanediol was retained from the data for testing biological activity in the field.

3.1.3. Biological activity (Fig. 2)

The ''bait'' and ''replicate'' effects were significant: $F(3,77)=22.02$; $P<0.0001$ and $F(2,77)=4.13$; $P<0.02$, respectively. Both factors significantly interacted: $F(6,77)=4.41$; $P<0.001$. The catches by the second replicate were responsible for the ''replicate effect'' and F1×F2 interaction. They were about twice as abundant than in the first and third replicates

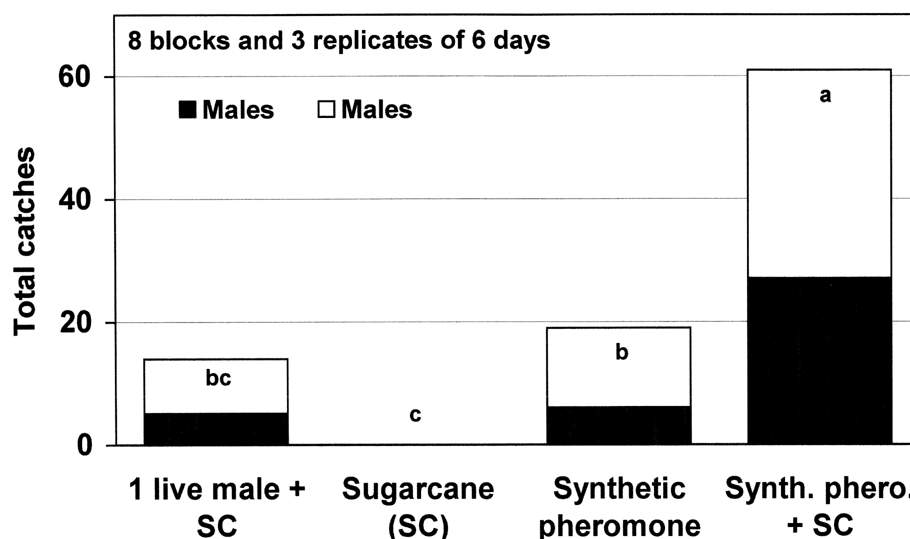

Fig. 2. Field catches of *S. australis* by a synthetic pheromone (Synth. phero.) mixture: (±)-2-butanol, 3-hydroxy-2-butanone and (±)-2,3-butanediol in 90:5:5 (v/v) ratio with or without sugarcane (SC) at Tavilo CCRI plantation (PNG; August 1998). The synthetic pheromone was emitted at 103±5 mg/h from open PE bottles (see Sections 2.1.3 and 2.4 for details). The mean catches per trap and per replicate associated to same letters do not significantly differ [Newman–Keuls' test at $\alpha=0.05$ on ln $(x+1)$ transformed data].

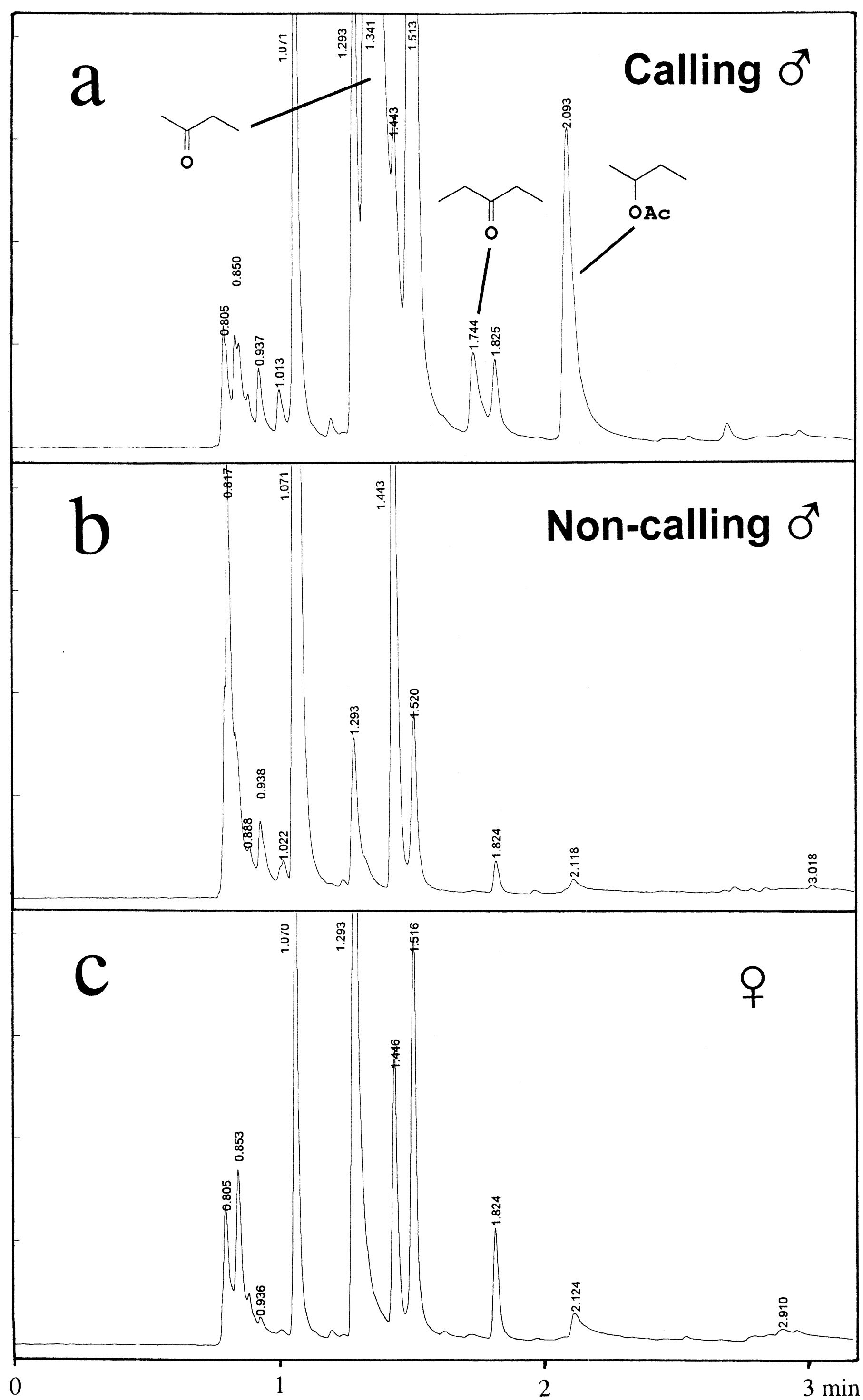

Fig. 3. Chromatograms of CAR–PDMS SPME-sampled volatiles from one calling *Strategus aloeus* male (a), one non-calling male (b) and one female (c) evidencing the three components of the male pheromone (sample origin and sampling conditions in Section 2.1.2; GC analysis on polar column under conditions **IV**; see Section 2.3.3).

and mostly recorded to "synthetic mixture+SC" while the "male+SC" controls caught no insect at all, contrary to what they did in the other replicates. The synthetic pheromone mixture was emitted at 103±5 (S.E.) mg/h (n=32). "Synthetic mixture+SC" caught significantly more beetles (>4×; total: 27♂, 34♀) than "male+SC" control (total: 5♂, 9♀) and sole SC (no catch). Catches by the sole synthetic mixture (total: 6♂, 13♀) and "male+SC" control were equivalent. Catches by the sole "synthetic mixture" were significant (different from zero: sole SC).

3.2. *Strategus aloeus male pheromone*

3.2.1. *Chemical identification*

The compared (polar and apolar columns) chromatograms of the CAR–PDMS SPME-sampled VOCs from controls (female and non-calling male in gallery) and calling *S. aloeus* males evidenced one major and two minor compounds characteristic of calling males (Fig. 3). EI and CI–NH_3 GC–MS data led us to tentatively identify these compounds as 2-butanone (major compound), 3-pentanone and *sec.*-butyl acetate, with M_r values of 72, 86 and 116, respectively. The identification was confirmed by comparison of retention times and mass spectral data with the reference molecules.

A mean of 0.34±0.06 (S.E.) μg pheromone per male with a 95.5:4.0:0.5 (w/w) ratios of 2-butanone, 3-pentanone and *sec.*-butyl acetate was sampled by CAR–PDMS SPME fibres. Analyses on the polar column led to similar results but the amount of *sec.*-butyl acetate could not be precisely estimated due to co-elution of two unidentified compounds. The w/w ratio of 2-butanone, 3-pentanone and *sec.*-butyl acetate in headspace at 1 cm from the insect abdomen could be estimated as 94:5:1 based on the K constants (see Section 3.2.3).

3.2.2. *Biological activity (Fig. 4)*

The beetles of both sexes spent more than half test time in the way odorised by the synthetic mixture on an average, whilst it represented 25% of the test area. Eleven beetles (6♂, 5♀) out of the 16 tested spent more than 25% time in the way odorised by the synthetic mixture, the choice of which was highly significant (P<0.0001).

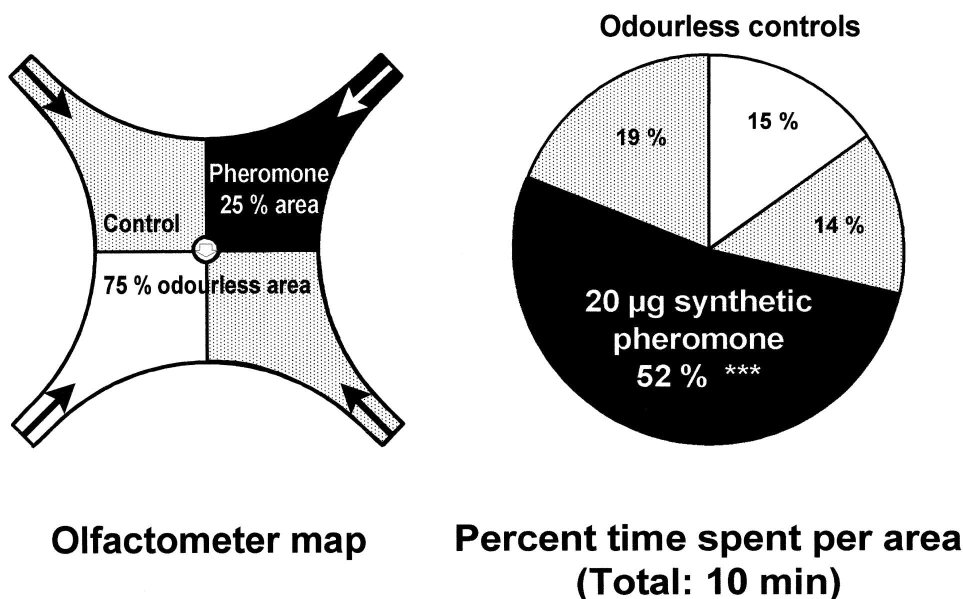

Fig. 4. Responses of *S. aloeus* (mixed sexes) to 20 μg doses of a synthetic pheromone mixture: 2-butanone, 3-pentanone and (±)-*sec.*-butyl acetate in 99.8:0.1:0.1 (v/v) ratio (aqueous solution) in a four-way olfactometer (July 1999). ***: The frequentation of the pheromone odorised way is significant (P<0.0001; binomial test with P=0.25; n=16; 9♂ and 7♀).

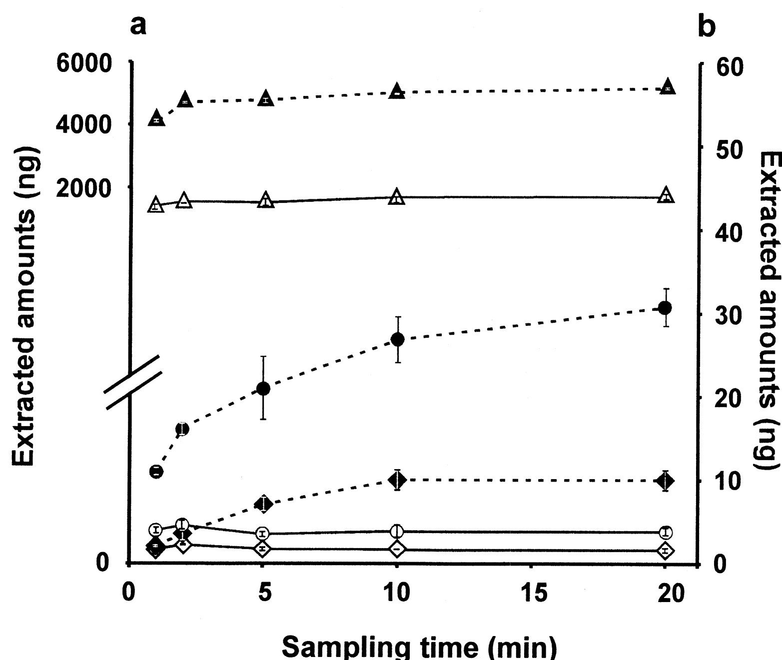

Fig. 5. Compared amounts of synthetic *Strategus aloeus* pheromone compounds extracted by PDMS–DVB (empty symbols–plain lines) and CAR–PMDS (filled symbols–dotted lines) SPME fibres as a function of the sampling times. Detailed conditions in Section 2.2.2. The bars associated with the symbols represent the standard deviations of the means. a (left scale): 2-butanone (triangles); b (right scale): 3-pentanone (circles) and *sec.*-butyl acetate (diamonds).

3.2.3. Compared SPME sampling parameters

The CAR–PDMS fibre exhibited 10- to 100-times greater K constants for 2-butanone, 3-pentanone and (±)-*sec.*-butyl acetate than the PDMS–DVB fibre exhibited for (±)-2-butanol and 3-hydroxy-2-butanone and (±)-2,3-butanediol (Table 1 and below). The CAR–PDMS fibre was twice as retentive for 2-butanone (K=107 ml) as (±)-*sec.*-butyl acetate (K=51 ml) and had an intermediate affinity for 3-pentanone (K=78 ml).

The comparison trial from the synthetic *S. aloeus* pheromone mixture confirmed that the CAR–PDMS fibre had greater adsorption capabilities than the PDMS–DVB fibre: twice to 10-times more pheromone chemicals were extracted by the first than the latter coating at any sampling time (Fig. 5). The extracted amounts reached plateaux from 2-min samplings for the PDMS–DVB fibre. The equilibrium times appeared longer with the CAR–PDMS fibre: from 10-min sampling the "fibre-headspace" equilibrium was reached for 2-butanone and (±)-*sec.*-butyl acetate but it was not for 3-pentanone (Fig. 5).

4. Discussion

The bioassays evidenced that the synthetic three-component mixtures reproducing the male-specific VOCs were attractive to both sex conspecifics. Thus *S. australis* and *S. aloeus* males produced aggregation pheromones that were closely related though the species belong to two distinct fauna spheres (Indo-Australian and Neotropical). *S. australis* pheromone was not based on pure enantiomers as frequently with Coleopteran pheromone [19–21], and racemic synthetic mixtures were bio-active in both species. This is the first report of Scarabaeidae pheromones based on such common molecules and with such low M_r values. The pheromones described in related Scarabaeidae belong to different chemical classes (mainly amino or fatty acid derivatives and ter-

penoids) and are much less volatile [19], especially the sole pheromone known in Dynastinae: ethyl 4-methyloctanoate (M_r 186) [8,9]. Highly volatile pheromones with M_r<100 are very rare in insects [19,21–24]. Methylated C_4 aliphatic alcohols and similar structures have been reported as minor aggregation pheromone components in Platypodidae and Scolytidae [21]. *n*-Butanol, acetic acid, *n*-, iso- and 2-methylbutyric acids are the smallest VOCs identified from structures involved in pheromone communication, especially scent hairpencils of male moths, but their actual pheromone role has never been clearly established in the concerned species [22].

Our work confirmed the usefulness of the SPME in the study of insect pheromones: the pheromones were isolated directly from a few live individuals in a rapid and reproducible way. SPME was an efficient alternative to work out *S. australis* pheromone after the negative results of conventional VOC trappings. The unusually high volatility of the pheromones contributed to the failure of the initial methodology.

The work was based on a pragmatic use of commercial SPME fibres. Isolating an unknown pheromone required the choice of a coating, which was a compromise between the coating properties and assumptions about the expected pheromone chemicals: highly to moderately VOCs [19,21–24]. Most SPME commercial coatings are suitable for extracting airborne VOCs. PDMS is better adapted to sample low to medium polarity VOCs [25]. It was successfully used to isolate Lepidoptera pheromones [4,5] and the pheromone of the weevil *Metamasius hemipterus* [3]. Mixed coatings are more suitable than monoconstituent ones for sampling VOCs because they associate complementary properties [25]. PDMS–DVB was retained for studying the Dynastid species because it allows to sample analytes within the broadest range of polarities and volatilities among the available coatings [25]. CAR–PDMS, which is more adapted to sample highly VOCs due to Carboxen properties was not used to study *S. australis* because it was not commercially available. It was further tested with success for *S. aloeus*. An initial comparative trial from the same calling males allowed us to rapidly select the CAR–PDMS rather the PDMS–DVB fibre to study this species. This decision was validated by the work with the synthetic pheromone in controlled conditions. The initial trial confirmed that CW–DVB coating, tuned to sample medium to high polarity and much less volatile chemicals, was not adapted (50 lesser VOC amounts collected than by the two first ones).

The availability of a large volume of pheromone secretion produced by *S. australis* helped in developing the isolation protocol. We operated in static controlled conditions to better fit the current practical recommendations for VOC sampling by SPME. Long times for secretion vaporisation and sampling, and a small volume for headspace formation were chosen because they optimised the probability to sample at equilibrium and detect the broadest range of VOCs [25].

This could not be realised with *S. aloeus* because the pheromone was emitted as a vapour and in peculiar conditions: a calling behaviour, only performed at the entrance of a gallery burrowed in soil. The 5-min samplings allowed one to reach equilibrium for two out of the three pheromone components but 3-pentanone proportion was likely underestimated. Longer samplings, initially retained, were abandoned because the insects either broke the SPME fibre or moved away from it.

The calibration (K) measurements were made from aqueous solutions to both better fit with the sampling from natural sources realised in moist air: *S. australis* pheromone secretion likely contained water and *S. aloeus* males emitted the pheromone from very humid soil, and to avoid adsorbtion of organic solvent with subsequent possible interference in the GC analyses (CAR–PDMS fibre has strong affinity for acetone and methylene chloride [14]). The air humidity indeed modify PDMS fibre affinities for hydrocarbons [26], which may also occur with other coatings and VOCs. No stirring nor silanised glassware were used because of the small volume sampled and the type of molecule studied (very low M_r). Stirring is important when sampling from aqueous solutions, from large volumes or when the analytes are emitted from heterogeneous matrix [25]. The adsorption capability of untreated glass surfaces that may bias K determination are negligible for short-chained hydrocarbons. They must be avoided with apolar long-chained VOCs on the contrary: Glass beads or capillaries were good moth pheromone traps for example [1]. No aberrant data were

recorded in our conditions except some erratic values for 2-butanone. The reasons for those were not investigated.

Because of its advantages SPME will certainly enable the study of semiochemicals in unexplored insect groups and contribute to new pheromone identifications. The recent availability of CAR–PDMS coatings has opened new possibilities to easily and efficiently sample highly VOCs of major biological significance.

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