

VOLATILE COMPOUNDS RELEASED BY DISTURBED FEMALES
OF *CEPHALONOMIA STEPHANODERIS* (HYMENOPTERA: BETHYLIDAE):
A PARASITOID OF THE COFFEE BERRY BORER *HYPOTHENEMUS HAMPEI*
(COLEOPTERA: SCOLYTIDAE)

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ABSTRACT

Volatile compounds released by disturbed females of the bethylid wasp *Cephalonomia stephanoderis* Betrem were collected and analyzed by gas chromatography-mass spectrometry. The origin of volatiles and their behavioral effects on conspecifics were also investigated. The source of the volatile compounds was found to be the head, and more specifically, the mandibular glands. These glands contain skatole as the main volatile component. Behavioral bioassays demonstrated that extracts of parasitoid heads and synthetic skatole evoked the same alarm behavior in this species. The possible function of this chemical is discussed.

Key Words: *Cephalonomia stephanoderis*, *Hypothenemus hampei*, alarm pheromone, skatole, biological control

RESUMEN

Los compuestos volátiles liberados por hembras molestadas del parasitoide betílido *Cephalonomia stephanoderis* Betrem fueron colectadas y analizadas por cromatografía de gases y espectrometría de masas. El origen de los volátiles y su efecto comportamental en los parasitoides conespecíficos fueron también investigados. La fuente de los compuestos volátiles fue localizada en la cabeza, y más específicamente, en las glándulas mandibulares. Estas glándulas contienen skatol como el principal componente volátil. Los bioensayos comportamentales demostraron que los extractos de cabezas del parasitoide y skatol sintético provocaron el mismo comportamiento de alarma en esta especie. Se discute la posible función de este compuesto químico.

Translation provided by the authors.

The bethylid wasp *Cephalonomia stephanoderis* Betrem (Hymenoptera: Bethylinidae) is an ectoparasitoid of larvae and pupae of the coffee berry borer, *Hypothenemus hampei* (Ferrari) (Coleoptera: Scolytidae), which is the most important pest of coffee worldwide (Barrera et al. 1990; Murphy & Moore 1990). *Cephalonomia stephanoderis* is native to Central West Africa and has been introduced to various coffee-producing countries (Murphy & Moore 1990). Adults of *C. stephanoderis* emit a strong odor when they are disturbed or transported to be released in field (Gómez 2005). This odor can be detected by the human nose (Infante et al. 2001). A number of parasitic wasps are known to emit more or less pungent odors (Townes 1939). The function of these odors in wasp behavior remains largely unknown; in some cases they have been thought to have a defensive role (Buckingham 1975) or to play an important role in courtship (Williams et al. 1988). However, only one *Cephalonomia* species, *C. gal-*

licola (Ashmead), a cosmopolitan ectoparasitoid of anobiid beetles, has been reported to release an odor when squashed by forceps (Kuwahara 1984). The odor originated from the head, and the chemical identified was skatole (3-methylindole). Infante et al. (2001) suggested that a similar secretion could be released by *C. stephanoderis*, but no studies have been carried out to identify the chemicals released by this species.

We describe here behavioral evaluation, origin, and identification of the volatile compounds emitted by adult female of *C. stephanoderis* when disturbed.

MATERIALS AND METHODS

Biological Material and Experimental Conditions

Adult *C. stephanoderis* were obtained from the laboratory colony maintained at El Colegio de la Frontera Sur, Tapachula, Chiapas, Mexico. Al-

though both sexes emit the odor (J. Gómez unpublished), females were chosen because of their importance as biological control agents and because the sex ratio is markedly biased in favor of females (Barrera 1994). The colony was established with insects collected from coffee plantations near Tapachula in 1999 and reared as described by Barrera et al. (1991). Bioassays were conducted in a room at $24 \pm 2^\circ\text{C}$, $80 \pm 5\%$ relative humidity and lit with red light (10 lux). Parasitoids used in the bioassays were collected from adult emergence jars on the day of the tests and placed in the bioassay room 3 h before testing.

Headspace Bioassay

Two groups of 20 *C. stephanoderis* females were placed in a separate glass vial (50 mm high \times 20 mm diameter) and a plastic vial (70 mm high \times 20 mm diameter) connected to each other by a plastic tube (Fig. 1). The first group of insects was strongly

shaken for 1 min. Preliminary observations showed that during this time the insects released the odor. The second group of insects was not disturbed. A disposable syringe was used to inject 35 ml of clean air into the glass vial in order to blow the volatiles through the tube on to undisturbed insects. Test insects were observed for any change in their behavior for 10 min after the influx of air. Alarm behavior was considered to happen if insects showed movements such as agitated running or attempts to take flight. No change in behavior was considered a lack of observable response. Clean air and the odor from undisturbed insects were used as controls. Six replicates per treatment were performed.

Bioassays with Extracts and Synthetic Skatole

Four different extracts were made with 20 and 40 heads, 40 thoraces, and 40 abdomens of *C. stephanoderis* females. Heads, thoraces, and abdomens were macerated in 200 μl of hexane. For eval-

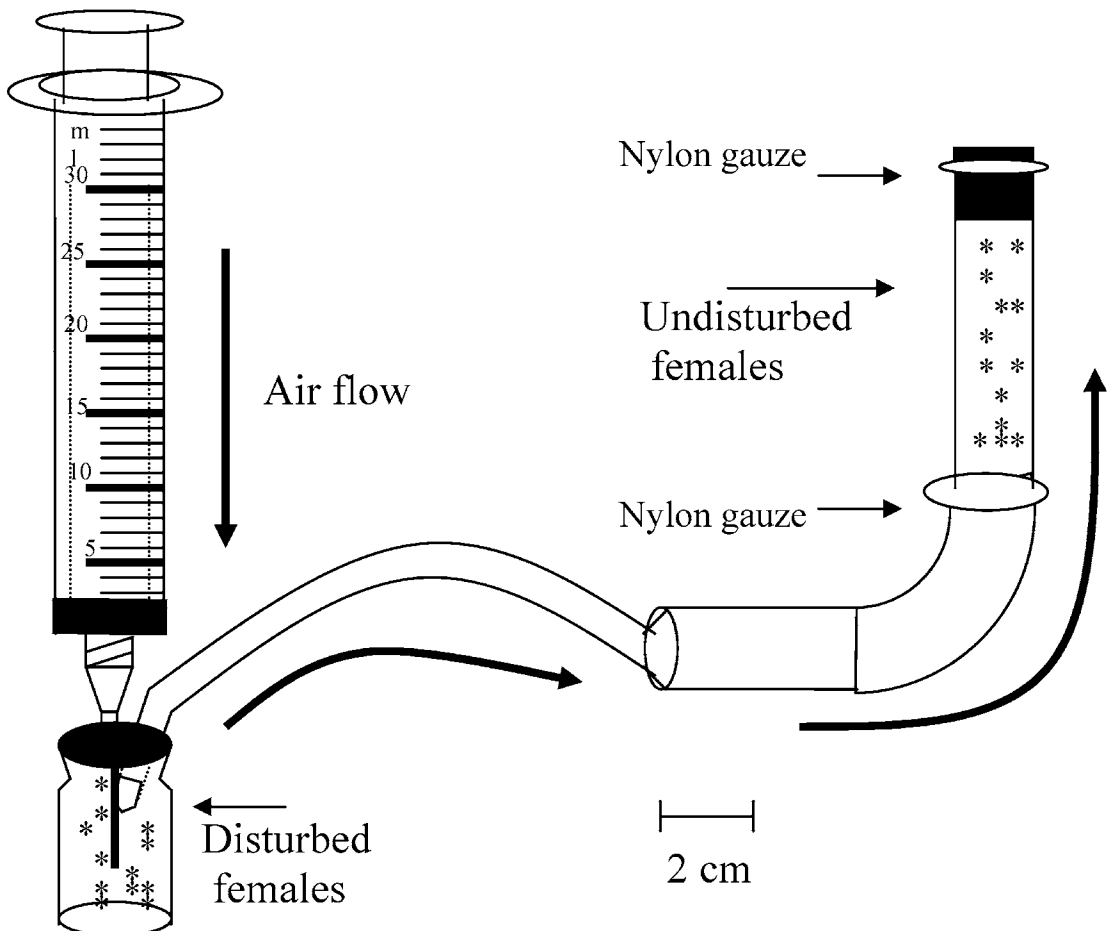


Fig. 1. Design of olfactometer used to determine the response of *C. stephanoderis* females to volatiles from disturbed and undisturbed insects and clean air.

uating the biological activity of these extracts, 10 μ l of the chosen extract (equivalent to one or two heads, two thoraces, or two abdomens depending on the extract used) was applied to a piece (1 cm²) of Whatman No. 2 filter paper. The solvent was allowed to evaporate for 1 min and then the piece of filter paper was placed in a Petri dish (10 cm diameter). An upside down glass tube (70 mm high \times 15 mm diameter) containing 20 undisturbed female parasitoids (prepared 2 h prior to the test) was placed over the filter paper. An alarm response was noted if the insects exhibited any movement. The number of insects showing alarm behavior was counted every 5 min for 40 min. In other experiments, several doses (0.1, 0.5, 1, 10, and 100 ng) of synthetic skatole in hexane were tested in the bioassay described above. Hexane alone was used as control in all experiments. Ten replicates were performed for all treatments. Skatole (98%) was obtained from Sigma Chemical, Co. (Toluca, Mexico).

Solid Phase Micro-Extraction (SPME)

SPME was conducted with a holder and a 100- μ m poly-(dimethylsiloxane)-coated fiber which were obtained from SUPELCO (Toluca, Mexico). Twenty *C. stephanoderis* females were placed inside a glass vial (7.5 mm high \times 1.5 cm diameter) with a foam cap. Sampling was performed by inserting the SPME needle, through the foam cap, into the headspace of the glass vial. Volatiles were allowed to be adsorbed onto the fiber for 5 min. Subsequently, it was removed from the vial and volatiles desorbed inside the heated injection port of a gas chromatograph for 5 min.

Solid Sampling Preparation for Chemical Analysis

Females of *C. stephanoderis* were killed by placing them in a refrigerator for 24 h. Dissections were made under a binocular microscope with fine forceps and entomological pins. Three heads, thoraces, and abdomens, and ten mandibles with the gland attached were dissected in distilled water and placed in thin-walled soft glass tubes previously sealed at one end; the open end was then sealed in a micro-flame for analysis by coupled gas chromatography-mass spectrometry (Morgan 1990). In addition, three whole insects were analyzed by this technique.

Chemical Analysis

Gas chromatography-mass spectrometry (GC-MS) was conducted with a Varian Star 3400 CX gas chromatograph linked to a Varian Saturn 4D mass spectrometer. The samples were analyzed in a fused silica column (30 m \times 0.25 mm) coated with poly-(5%-diphenyl-95%-dimethylsiloxane) programmed from 50°C to 250°C at 15°C/min. The flow rate of helium through the column was main-

tained at 1 ml/min. The injector port temperature was held at 200°C. The glass capillaries containing either the glands, heads, abdomens, or thoraces were directly inserted into the injection area and heated and crushed as described by Morgan (1990).

Scanning Electron Microscopy

Mandibular glands of *C. stephanoderis* females were washed and fixed in a solution of 3% glutaraldehyde in phosphate buffer (0.1 M, pH 7.2). Glands were washed twice for 5 min with distilled water, and then passed through increasing grades of ethanol, from 10% to absolute ethanol, 30 min each. Finally they were dried to critical point of CO₂, mounted in aluminum stubs and sputter coated with gold-palladium (Dykstra 1993). The samples were examined and photographed in a Topcon SM-510 electron microscope operated at 5 kV.

Statistical Analysis

Results were subjected to one-way analysis of variance (ANOVA) (SPSS for Windows 8.0. SPSS Inc.), except the data for insect response to head extracts and skatole over time were analyzed by repeated measures ANOVA. When *F* values were significant, means were compared by Tukey's test at $\alpha = 0.05$.

RESULTS

Headspace Bioassay

Undisturbed *C. stephanoderis* females showed a significant response to volatiles from disturbed females compared with the response to volatiles from undisturbed females and clean air ($F = 85.5$; $df = 2, 15$; $P < 0.001$). From a total of 120 insects tested, 81 females were affected by the odor from disturbed females; the rest (39) remained stationary. In contrast, most of the individuals remained stationary when clean air and air from undisturbed insects (111 out of 120 in each treatment) was passed over the parasitoids.

Female Response to Extracts

Females showed a significant alarm response (e.g., agitated running) to head extracts, but not to thorax extracts or abdomen extracts or hexane control ($F = 90.7$; $df = 4, 45$; $P < 0.001$) (Fig. 2). Of the total of females tested, 64% were disturbed when a one-head equivalent extract was introduced on the filter paper, whereas 80% were disturbed when a two-head equivalent extract was used.

Chemical Analysis

The SPME and GC-MS analysis of the parasitoid volatiles showed that agitated *C. stephanoderis* females released at least two compounds (Fig.

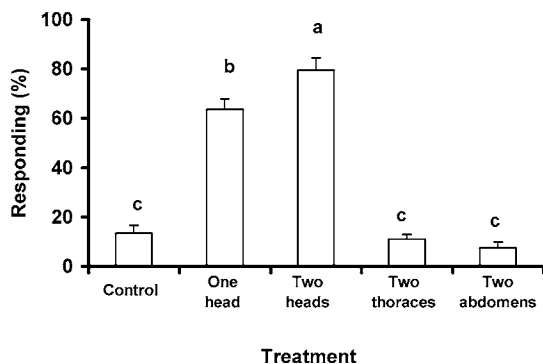


Fig. 2. Percentage of *C. stephanoderis* females that responded to extracts containing equivalent of one or two heads, two thoraces, or two abdomens. Vertical bars indicate the standard error of the mean. Different letters over bars indicate that means are significantly different (ANOVA, followed by Tukey test, $P < 0.05$)

3a). Compound 1 was identified as skatole (3-methylindole) by comparison of retention time and mass spectrum with that of the synthetic standard. Compound 2 with a Kovat's Index of 15.95 showed mass spectrum fragment ions at m/z 55 (80%), 69 (70%), 83 (25%), 97 (100%), and 111 (25%). This mass spectrum resembled that of a branched alkene. Undisturbed insects did not release skatole or compound 2 (data not shown). Solid sampling analysis of heads of *C. stephanoderis* showed that the volatiles contained a mixture of hydrocarbons and nitrogen compounds (Fig. 3b). The compounds detected were skatole, unidentified compound 2, and other nitrogen compounds which were tentatively identified by mass spectral matching to a library data base (NIST 2002) as (3) uric acid, (4) dl-alanyl-leucine, (5) hexahydro-3-[2-methylpropyl]pyrrolo [1, 2-a] pyrazine-1, 4-dione and (7) oleamide. Compounds 6, 8, 9, 10, 11, 12, 13, 14, 15, and 16, which were common in all solid samples were cuticular in origin as confirmed by analysis of a small fragment of cuticle. They have mass spectra typical of hydrocarbons, which have been identified previously from *C. stephanoderis* by Howard & Infante (1996). The analysis of the mandibular glands showed that one of the components of the glands was skatole with traces of all the other compounds found in the head (Fig. 3c). Analysis of thoraces and abdomens showed that these contained the compounds 3, 4, 5, and 7, and hydrocarbons but not skatole or unidentified compound 2 (Figs. 4d and 4e), confirming that compounds 1 and 2 are found specifically in the head. Skatole content in the head varied from 0.4 to 1.0 ng ($n = 6$; 0.5 ± 0.1 SE).

Female Response to Synthetic Skatole

The alarm behavior of *C. stephanoderis* was significantly influenced by the dose of synthetic

skatole ($F = 5.0$; $df = 4, 45$; $P = 0.002$). Multiple comparisons indicated that the dose of 1 ng skatole elicited significantly larger alarm behavior compared with those evoked by the doses of 0.1 and 100 ng of this compound. The alarm responses elicited by the doses of 0.5 and 10 ng of skatole were intermediate between and not significantly different from those evoked by the doses of 1, 0.1, 100 ng of this compound (Fig. 4).

The wasp response to head extracts and skatole over time revealed that the type of chemical stimuli used did not influence differently the alarm behavior of *C. stephanoderis* ($F = 1.23$; $df = 2, 27$; $P = 0.307$). In contrast, time affected the wasp response to the chemical stimulus ($F = 6.34$; $df = 7, 189$; $P < 0.001$). In the three treatments, the parasitoids started to respond soon after the samples were delivered to the vial reaching the highest response at 15-20 min (35 min in the case of skatole), and after this time the insect response gradually declined (Fig. 5). The chemical stimulus \times time interaction term was not significant ($F = 1.24$; $df = 14, 189$; $P = 0.25$).

Scanning Electron Microscopy

The microphotograph showed that the mandibular gland is connected to the base of the mandible (Fig. 6a). The gland is comparable in size to the mandible. A close-up shows that the gland consists of a series of tubular structures attached to the mandible (Fig. 6b).

DISCUSSION

The alarm behavior of *C. stephanoderis* was observed in individuals that were exposed to the headspace volatiles collected from disturbed females. No such behavior was observed in wasps exposed to the volatiles collected similarly from undisturbed females. SPME and GC-MS analysis showed that skatole was the main volatile compound emitted by disturbed females. The presence of skatole has been reported in two species of Neuroptera (Blum & Wallace 1973), one species of Trichoptera (Blum 1981), one species of Coleoptera (Burger et al. 2002) and several species of Hymenoptera (Law et al. 1965; Smith & Roubick 1983; Kuwahara 1984; Keegans et al. 1993; Billen et al. 1998). For instance, skatole was found in the ant *Pheidole fallax* Mayr, although its function was not determined (Law et al. 1965). This compound is released from the abdomen of the army ant, *Labidus praedator* (Smith) and functions as a trail pheromone (Keegans et al. 1993). The mandibular gland of a leptanillan ant, *Leptanilla* sp. contains a large amount of skatole that is released as an alarm pheromone (Billen et al. 1998). Skatole is present in the mandibular gland of a stingless bee, *Melipona interrupta triplaridis* Schwarz as a component of the alarm pheromone (Smith &

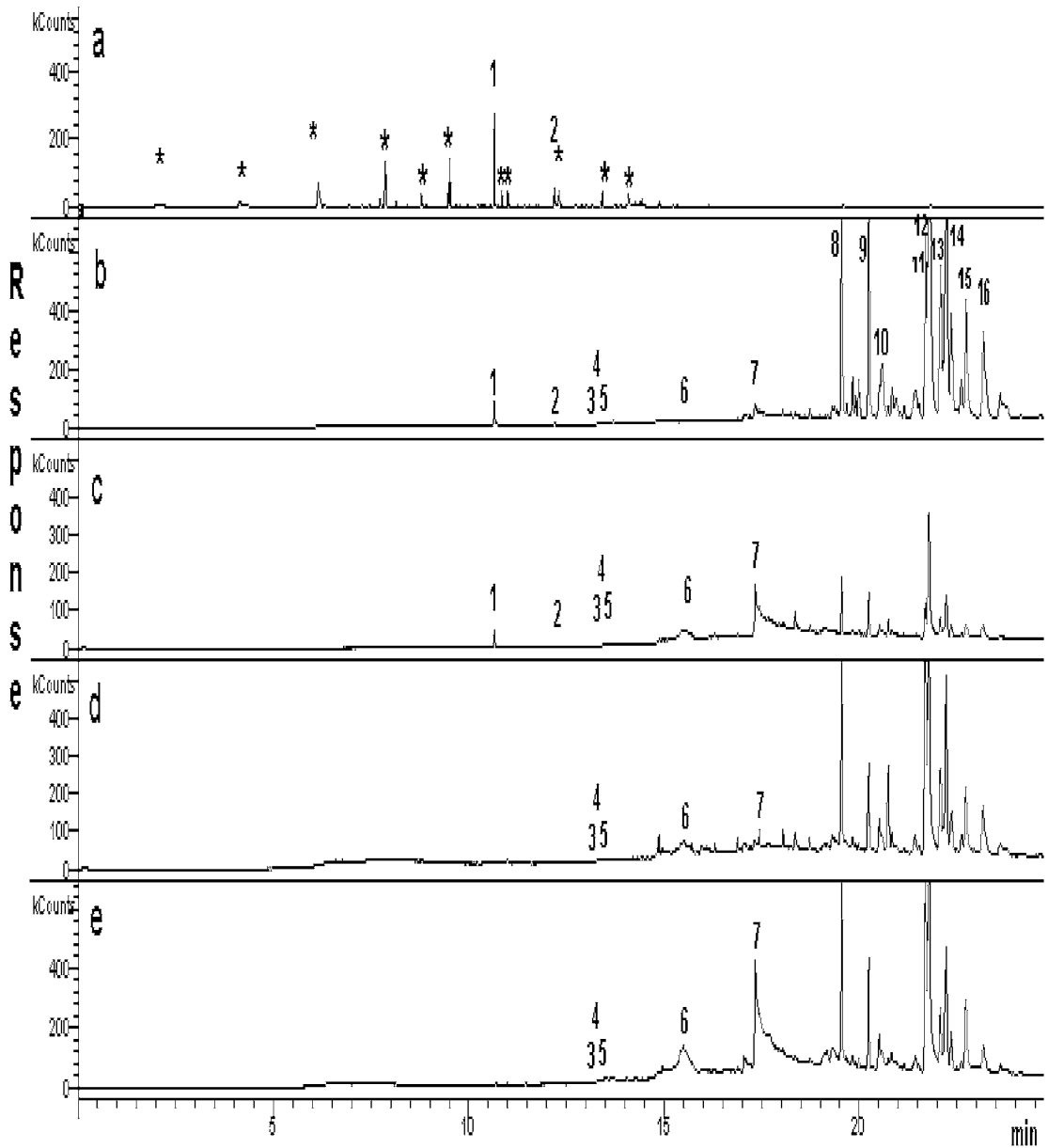


Fig. 3. Representative chromatograms of compounds released by disturbed females collected by SPME (a); and solid sampling analysis of crushed heads (b), mandibular glands (c), abdomens (d), and thoraces (e) of *C. stephanoderis* females. Skatole (1), unidentified (2), uric acid (3), dl-Alanyl-l-leucine (4) hexahydro-3-[2-methylpropyl]-pyrrolo [1,2-a] pyrazine-1, 4-dione (5), oleamide (7) and hydrocarbons (6), (8), (9), (10), (11), (12), (13), (14), (15) and (16). *Contaminants from the solid phase fiber.

Roubik 1983). Kuwahara (1984) detected skatole in the head of *C. gallicola*, another bethylid wasp, and proposed a function as an allomone.

Female parasitoids of *C. stephanoderis* showed the highest alarm response to one and two-head equivalent extracts and 1 ng of skatole. However, response was reduced to higher doses of skatole,

which may indicate that higher doses of skatole may disrupt the effective alarm communication between parasitoids, as has been found in aphids (El-Agamy & Haynes 1992). A high dose of (*E*)- β -farnesene, the aphid alarm pheromone, produced a rapid sensory habituation of aphids to this compound (Calebrese & Sorenson 1978). In our study,

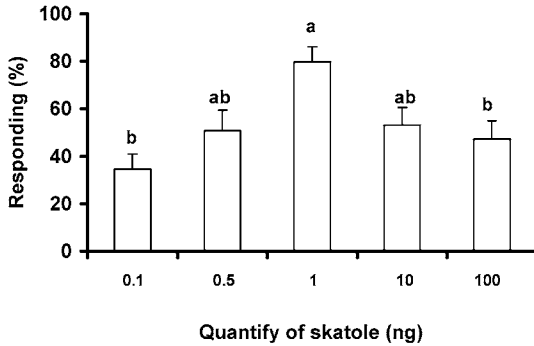


Fig. 4. Percentage of *C. stephanoderis* females that responded to different quantities of skatole. Vertical bars indicate the standard error of the mean. Different letters over bars indicate that means are significantly different (ANOVA, followed by Tukey test, $P < 0.05$).

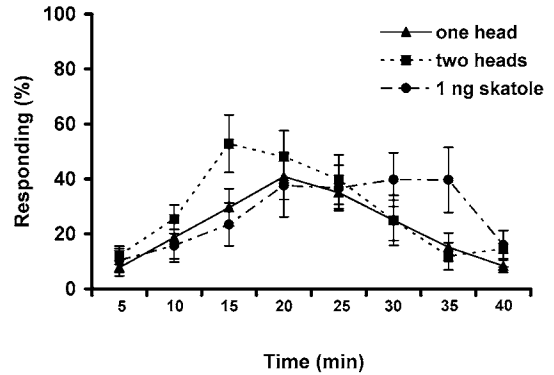


Fig. 5. Percentage of *C. stephanoderis* females that responded to one or two-head equivalent extracts and skatole (1 ng), at different times of observation. Vertical bars indicate the standard error of the mean.

the stimulatory effect of head extracts or skatole was short-lived (<35 min); therefore the decrease in the alarm response of *C. stephanoderis* may be due to sensory adaptation or habituation.

The odor released by agitated adults of *C. stephanoderis* may have multiple functions as reported for other insects (e.g., Blatt et al. 1998; Staples et al. 2002; Wardle et al. 2003). In this study, we analyzed only females, but preliminary studies have indicated that male parasitoids also release the same compounds (unpublished data). The fact that both sexes produce the same components in the secretion suggests that they do not function as sexual pheromones. Generally, defensive secretions are released by both sexes as has been demonstrated for bugs (Leal et al. 1994), thrips (Teerling et al. 1991), and cockroaches (Farine et al. 2002). For example, the defensive secretions of the glandular pouches of the adults of both sexes of cockroaches *Thera petiveriana*

(L.) contain volatile compounds that function as an alarm pheromone for adults (Farine et al. 2002). A potential function for skatole in *C. stephanoderis* is as an alarm pheromone causing dispersal. *Cephalonomia stephanoderis* adults are found in groups of sisters and brothers inside coffee berries after they emerge from the cocoon and they remain together 4-5 days to mate before they disperse (Barrera et al. 1989). A pheromone could promote the dispersion of the parasitoids after mating. A prerequisite to the evolution of alarm pheromones is the evolution of sociality (Nault & Phelan 1984). Another possible function of skatole in *C. stephanoderis* is as an epideictic pheromone, promoting spacing in the natural habitat (Haynes & Birch 1985). *Hypothenemus hampei*, the host of *C. stephanoderis*, reproduces inside coffee fruits, which may represent a limited resource for both coffee berry borer and parasitoids, thus an epideictic pheromone could well be ad-

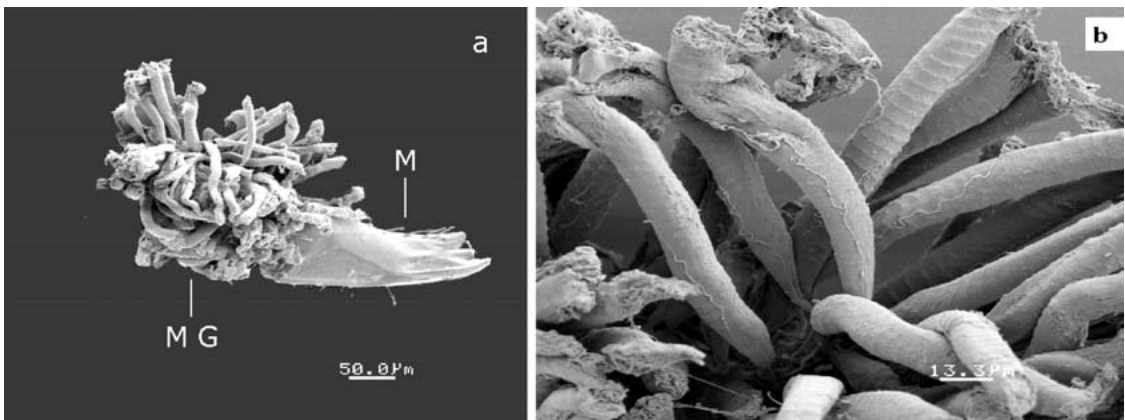


Fig. 6. Scanning photomicrograph of the mandibular gland attached to the mandibula of *C. stephanoderis*, M = mandible; MG = mandibular gland (a), Close up of the tubular structure of the gland (b).

aptative for individuals to reduce competition for resources. Barrera et al. (1994) presented evidence of a marking pheromone in *C. stephanoderis* to avoid use of hosts previously parasitized, but this possible pheromone seems to act over short distances compared to the odor released by agitated adults. A third possible function is that the odor is released in direct response to threats. Several species of spiders and ants have been reported to attack to *C. stephanoderis* as well as a bethylid wasp, *Prorops nasuta* Waterston in the coffee plantations of Mexico (Henaut et al. 2001; Infante et al. 2003). Some ant species occasionally forage inside coffee fruits and prey upon immature and adult *P. nasuta* and possibly *C. stephanoderis* (Infante et al. 2003). The hyperparasitoid *Alloxysta brevis* (Thompson) applies defensive compounds stored in mandibular gland reservoirs against attacking ants and other generalist predators like spiders (Hubner & Dettner 2000). Finally, the odor could mediate the interactions between *C. stephanoderis* and two other species of bethylid parasitoids of the coffee berry borer, *P. nasuta* and *C. hyalinipennis* Ashmead. Female parasitoids actively defend parasitized hosts and their progeny when intruders attempt to take possession of these resources (Pérez-Lachaud et al. 2002). Thus, it is possible that the odor of *C. stephanoderis* could function as an alarm pheromone, epideictic pheromone, or allomone depending on the specific situation. For example, the same compounds that function as an alarm pheromone in the bedbug, *Cimex lectularius* L., also serve a defensive role, as they can effectively repel *Monormorium pharaonis* (L.), a natural enemy of bedbugs (Levinson et al. 1974). These glandular secretions also make bedbugs distasteful to bat species which are predators to *C. lectularius*. It is believed that this defensive role may have been the primary function of the secretion and the alarm response of conspecifics evolved secondarily (Nault & Phelan 1984).

Our microphotographs of the mandibular gland of female *C. stephanoderis* show that the gland is not typical of Hymenoptera, which have been shown to contain a reservoir in the form of a sac (e.g., Cruz-Landim 1990; Mayhe & Caetano 1994). The mandibular gland secretion in *C. stephanoderis* presumably is produced by the tubular structures attached to the mandible.

In conclusion, this study showed that the behavior of female wasps of *C. stephanoderis* was affected by the introduction of volatiles released from disturbed conspecific females. The source of the volatile compounds was found to be the head, and more specifically, the mandibular glands. These glands contain skatole as the main volatile component. Behavioral bioassays demonstrated that extracts of parasitoid heads and synthetic skatole evoked the same alarm behavior in this species. An unidentified compound 2 released by

agitated females should be properly identified and its biological activity evaluated alone and in combination with skatole. Other unidentified compounds found in the heads and mandibular glands beside skatole may not be involved in the alarm behavior of *C. stephanoderis* because they were not detected in the headspace volatile analysis.

ACKNOWLEDGMENTS

We thank Francisco Infante and Trevor Williams (ECOSUR) for helpful reviews of the manuscript, Javier Valle-Mora for statistical advice, and Guadalupe Nieto for the scanning photomicrographs. J. Gómez received a doctoral grant from CONACyT.

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