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Research

Volatile Compounds Release by the Hair Pencils in Male *Prophantis smaragdina* (Lepidoptera: Crambidae: Spilomelinae)

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Abstract

Courtship behavior in several pyralid species is associated with the exposure to male hair pencils (HPs) or special scales that released volatile compounds. HP chemicals induce conspecific female and/or male behaviors and are therefore qualified as male pheromones. Preliminary observation on the coffee berry moth (CBM), *Prophantis smaragdina* Butler (Lepidoptera: Crambidae: Spilomelinae) mating behavior showed that the male displays abdominal HPs located on the last abdominal segment. The aim of the study was to identify the male volatile compounds and assess the results by electroantennography (EAG) on male and female antennae. Gas chromatography coupled to mass spectrometry analysis of male HP emissions showed five aromatic compounds identified as phenylacetaldehyde, phenylethyl alcohol, creosol, perillyl alcohol, and methyl anthranilate. EAG results showed that creosol elicited a significantly higher response than the control (hexane) on both male and female antennas. On female antennas, response to methyl anthranilate and phenylacetaldehyde was also significantly higher than the response to the control. Those results suggest that the creosol could play a behavioral role on conspecific male and female CBM during courtship behavior and mating choice. Methyl anthranilate and phenylacetaldehyde could also play a role on female behavior. Perillyl alcohol is for the first time identified as an insect product.

Key words: GC-MS, EAG, mating behavior, male hair pencil compound, Methyl anthranilate

Moth mating behavior relies on male attraction by the female sex pheromone. After locating the female and landing in the vicinity, the male undertakes a courtship more or less sophisticated, leading to a copulation attempt and female acceptance. In several species, the courtship behavior in male moths is associated with the display of scent-releasing organs called androconial scales, scent fans, hair pencils (HPs) or coremata, from which volatile compounds are emitted (Birch et al. 1990). The females detect the compounds during the courtship and a kind of chemical dialog is established between the male and the female (Baker 1983, Jacquin et al. 1991). HP chemicals were identified in several moth species as a blend of volatile molecules, often aromatic molecules (Aplin and Birch 1970, Blum 1987, Birch et al. 1990). Male HPs are diverse across taxa and are not constant within a systematic group. HP volatile compounds communicate information specific to a species and play a major role in reproductive isolation between sympatric, closely related species (Hillier and Vickers 2011). HP components act on conspecific female and/or male behaviors and can be considered as male pheromones. They influence the overall mating success of a courting male (Fitzpatrick and McNeil 1988, Hillier and Vickers 2004), and a significant decrease in mating success was observed when HPs were extruded or washed with solvent solution (Hirai 1977, Jacquin et al. 1991, Lassance and Löfstedt 2009, Roscoe et al. 2016). Although the precise role of the HP-associated pheromones may vary from one species to another and from one author to another, the previously cited authors conclude that male pheromones are critical for female acceptance (Birch et al. 1989). In the Oriental fruit moth *Grapholita molesta* Busk (Lepidoptera: Tortricidae), males display the HPs in front of the females and attract them at short range (Nishida et al. 1982, Baker and Haynes 1989). Such a behavior was also observed in *Mamestra brassicae* L. (Lepidoptera: Noctuidae) (Frérot, unpublished data). Male moth pheromones are also described as affecting the behavior of conspecific males, inducing an attraction Baker (1983) or an inhibition (Hirai 1977). Some species, in response to conspecific male HP emissions, aggregate such as lekking behavior in male tobacco moths *Ephestia elutella* Hübner (Lepidoptera: Pyralidae) (Phelan et al. 1986).

Coffee berry moth (CBM), *Prophantis smaragdina* Butler (Lepidoptera: Crambidae: Spilomelinae) is an important coffee pest mainly present in tropical and subtropical African countries and in

some islands in the south-western Indian Ocean (Waller et al. 2007, Guillermet 2009). Preliminary observations showed that females initiate the calling behavior between three and a half and nine hours after the beginning of the scotophase and mating occurs between four and nine hours after the beginning of the scotophase (Chartier, Lavogez, unpublished data). The male exhibited a very short courtship (less than 2 s) for a successful copulation, whereas for unsuccessful mating it lasts longer because of the replication the male attempts to copulate. When close to the female, the male curves the abdomen and attempts to copulate. At this stage, male abdominal HPs are displayed and a fragrance can be smelt by the human nose. These preliminary observations suggest that the male CBM emitted volatile compounds that may act as a pheromone during the courtship behavior. When mating has occurred, the pair assumes a 'tail to tail' position.

The present paper described identification of five male HP volatile compounds using gas chromatography coupled to mass spectrometry (GC-MS) and electroantennography (EAG) on female and male CBMs.

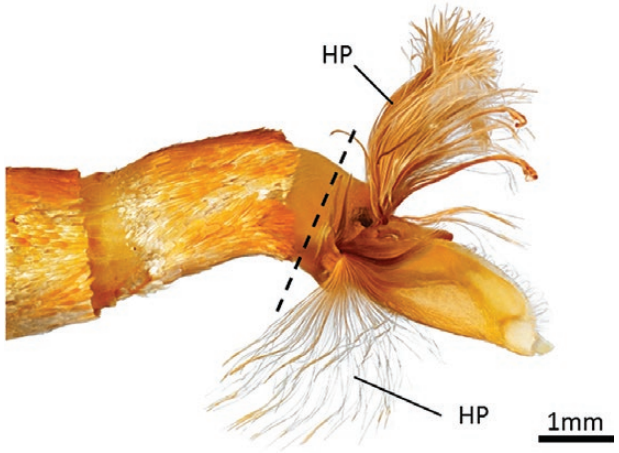

Fig. 1. Lateral view of male genitalia of coffee berry moth extruded using gentle pressure at the extremity of the abdomen. The dotted line represents the cut for extractions. HP= hair pencils. Camera: AZ-100 Nikon. Source: A. Franck (CIRAD).

Materials and Methods

Insect Rearing

Imagos used for our experiments came from field-collected larvae which were reared in the laboratory. Green coffee berries (*Coffea* spp.) were harvested during the fructification phase every 1 to 2 wk on six different cultivars in the coffee international germplasm collection of the French Development Research Institute (21°18′S, 55°30′E) and on 'Bourbon Pointu' cultivar in five fields (21°04′S, 55°18′E for two fields; 21°14′S,55°30′E; 21°12′S, 55°20′E and 21°04′S, 55°18′E) on Reunion Island. Coffee berries were spread on plastic-lid butter dishes (25 × 15 cm) and put inside a 30-cm fine-mesh nylon net cubic cage kept at 25 ± 1°C, 60 ± 10% relative humidity, and 12:12 L:D photoperiod. In the field, final instar CBM larvae fall on the ground and spin a cocoon with a folded dry leaf for the prepupa and pupa stages. To reproduce this in the laboratory, double layers of paper towel were put into the cage under the elevated lid of the butter dish containing the berries allowing the larvae to pupate. Every week, rotten and dry berries were removed and replaced. Pupae were collected, sexed, and put separately in 30-cm fine-mesh nylon net cubic cages with 2% sucrose solution under the same temperature and relative humidity conditions until emergence. Female and male cages were kept in two separate rooms to avoid odor experiences between the two sexes.

Chemicals

Synthetic chemicals methyl 2-aminobenzoate (methyl anthranilate) (>99%), *p*-mentha-1,8-diene-7-ol (perillyl alcohol) (≥95%), 2-phenylacetaldehyde (phenylacetaldehyde) (>90%) and 2-methoxy-4-methylphenol (creosol) (>98%) were purchased from Sigma Aldrich (St-Quentin Fallavier, France).

Extractions of Male HP Volatile Compounds for GC-MS

Preliminary tests showed a better GC-MS detection of male HP volatile compounds after collection on Solid Phase MicroExtraction (SPME) fibre extraction than after solvent extraction (Frérot et al. 1997). HP volatile compounds were collected on SPME fibres according to the protocol described by Ameline (1999), during the day. HPs from one male of 3–10 d (Fig. 1) were excised and placed individually on a drop of physiological solution on the wall of a 2-ml vial. An SPME fibre was inserted into the vial for 2 h then removed from the vials and kept at –4°C before analysis. Simultaneously, five heads were in hexane (200 µl) as control. Four replications of each collection were prepared.

Male HP Extraction for EAG

For EAG, five excised pairs of HPs from 3- to 10-d-old males were put in 200 µl of hexane and keep at –4°C until use.

GC-MS Analysis

SPME fibres containing HP volatiles and head extracts were analyzed using a Bruker Scion 436-GC linked to a Bruker Scion SQ detector. The oven was equipped with a fused silica capillary column (30 m × 0.32 mm i.d.) Rxi-5SilMS (0.25-µm film thickness, Restek) and was programmed from 50 to 300°C at 8°C/min. The carrier gas was helium N60 at constant flow of 2 ml/min. Each SPME fibre was manually inserted in the injector and the HP compounds desorbed in splitless mode, 5 min at 250°C. Mass spectra were recorded in electron impact mode at 70 eV. Kovats' retention indexes (RI) were computed using n-alkane solution from C10 to C24, eluted under the same conditions as the samples. HP compounds were identified according to their RI and to their mass spectra compared with the INRA library of synthetic compounds and NIST 2014 library (NIST 2014). HP compounds which were also detected in head extracts were not further selected.

Electroantennography

EAG is an effective tool to evaluate and record the olfactory responses of insects although the technique reflects only the antennae nerve activity. The insects aged 3- to 10-d old were immobilized using a 1-ml micropipette tip cut at the extremity to allow the head to pass through. EAG electrodes were filled with Ringer solution. The reference electrode was inserted into the eye, and the recording electrode covered the antenna tip whose distal part was removed. Tested synthetic chemicals were impregnated onto a strip of filter paper (10 × 5 mm), and then placed in a disposable glass pipette. While the air was continuously blown over the head of the moth (40 ml/s), the pipette tip containing the stimulation was inserted in a hole at the end of the glass tube carrying the air stream and an air puff was sent inside this pipette for 0.5 s at 12 ml/s for the stimulation. The signals

were amplified and connected to Labview (Labview 5.1, National Instruments, USA) for electrophysiological trace observation. The response of the antennae was expressed in mV. Six treatments were compared: the control was 1 µl of hexane, 10 µl of HP extract, methyl anthranilate, perillyl alcohol, phenylacetaldehyde, and creosol at 1 µg/µl were blown once on five different female antennas and on four different male antennas with an interval of at least 1 min between successive stimulations. The order of the six treatments was randomly changed for each antenna.

Statistical Analysis of EAG Data

The comparison of the antennal responses elicited by the treatments was carried out using a linear mixed model. The treatments, the sex, and the treatment × sex interaction were considered as fixed effects. The order of the treatment within each antenna was added as a fixed effect quantitative covariate, to take into account a possible change in antenna response to stimulus with time. The sex × succession order was nonsignificant and therefore discarded from the model. Antennae were considered as a random replication effect. The analysis was carried out using SAS procedure MIXED (SAS Institute 2008). The comparison was carried out by computing the differences of least squared mean, and the significance of differences was tested using a *t*-test, using the procedure MIXED (with the LSMEANS instruction). As the sex effect appeared nonsignificant, it was discarded from the model. As the treatment × sex interaction appeared significant, each treatment was compared to the control within each sex. The depolarization data were submitted to a Log transformation before analysis to achieve a normal distribution of the residuals of the model.

Results

GC-MS Analysis

GC-MS analysis of male HP emission showed five compounds (Fig. 2) identified according to the NIST 2014 library (NIST 2014) and confirmed by analyzing the synthetic molecules in the same GC-MS conditions. Comparison of the RI computed with the retention time of external standard made with C10 to C22 hydrocarbons (Table 1), and mass spectra confirmed the identity of each component. Two minor compounds were identified as: phenyl acetaldehyde about 4% (1) (CAS Number 122-78-1) (M+ = 120, 12.5%; m/z = 91 [100%], 65 [30%], 51 [8%], 39 [17%]) and 2-phenyl ethanol about 7% (2) (phenylethyl alcohol, CAS Number 60-12-8), (M+ = 122, 10%, m/z = 91 [100%], 65 [30%], 51 [8%], 39 [18%]). Three main compounds were identified as: creosol, about 20% (3) (2-Methoxy-4-methylphenol, CAS Number 93-51-6) M+ = 138, 75%, m/z = 123 (100%), 95 (67%), 67 (75%), 39 (63%); perillyl alcohol, about 25% (4) (p-Mentha-1,8-diene-7-ol, CAS Number 18457-55-1), M+ = 152 (5%), m/z = 39 (100%), 121 (25%), 91 (63%), 79 (75%), 76 (75%), 53 (50%); and methyl anthranilate, about 27% (5) (Methyl 2-aminobenzoate, CAS Number 134-20-3) M+ = 151 (50%), m/z = 119 (100%), 92 (75%), 65 (38%) 39 (12%).

EAG Recording

Male HP extract and four out of the five identified aromatic molecules: methyl anthranilate, perillyl alcohol, phenyl acetaldehyde, and creosol were tested by EAG on male and female antennas (Fig. 3). The EAG responses were significantly different among the tested compounds ($F= 23.13$; df =5, 33; $P < 0.001$). The interaction between chemical compounds and the sex of the insect was also significant ($F= 3.63$; df= 6, 33; $P= 0.007$). However, the amplitude of antennal response was not significant between male and female ($F=0.30$; df = 1, 7; $P = 0.603$).

Male HP extract caused a significantly higher antennal response than the control on both female and male antennas ($P <0.001$ on both male and female). Among the four synthetic molecules tested by EAG, only creosol response was significantly higher than the control and equivalent to the response elicited by male HP extract on both female and male antennas ($P <0.001$ on both male and female). On female antennas, response to methyl anthranilate and phenylacetaldehyde was significantly higher than the response to the control (respectively, $P = 0.0328$ and $P = 0.0263$) but perillyl alcohol response did not ($P= 0.1179$). On male antennas, methyl anthranilate, phenylacetaldehyde, and perillyl alcohol responses were not significantly higher than the response to the control (respectively, $P = 0.8957$, $P= 0.1493$, and $P = 0.6446$).

Discussion

The HPs of CBM released a blend of phenylacetaldehyde (9%), 2-phenyl ethanol (3%), creosol (37%), perillyl alcohol (18%),

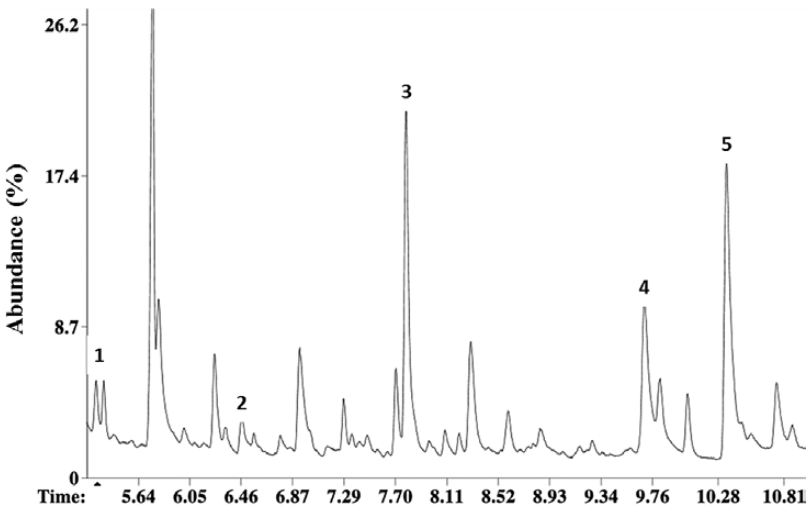

Fig. 2. Total ion current chromatogram of a hair pencil SPME fibre extract. 1: phenyl acetaldehyde, 2: 2-phenyl ethanol, 3: creosol, 4: perillyl alcohol, 5: methyl anthranilate.

Table 1. Mean ratio, retention times (RT), and retention indexes (RI) of the five aromatic compounds found in male hair pencil SPME fibre extracts ($N = 4$ hair pencil SPME fibre extracts)

Molecule names	Ratio (%)		RT		Synthetic	RI		Synthetic
			Extract			Extract		
Phenyl acetaldehyde	4.51	± 4.32	5.25	± 0.05	5.35	1,043.40	± 2.27	1,041.9
2-phenyl ethanol	7.40	± 3.57	6.40	± 0.06	6.307	1,113.00	± 2.88	1,114.5
Creosol	20.98	± 9.55	7.71	± 0.07	7.62	1,189.03	± 2.91	1,192.1
Perillyl alcohol	25.80	± 9.95	9.59	± 0.08	9.47	1,301.35	± 2.93	1,301.9
Methyl anthranilate	27.01	± 21.25	10.26	± 0.06	10.20	1,342.53	± 4.42	1,344.3

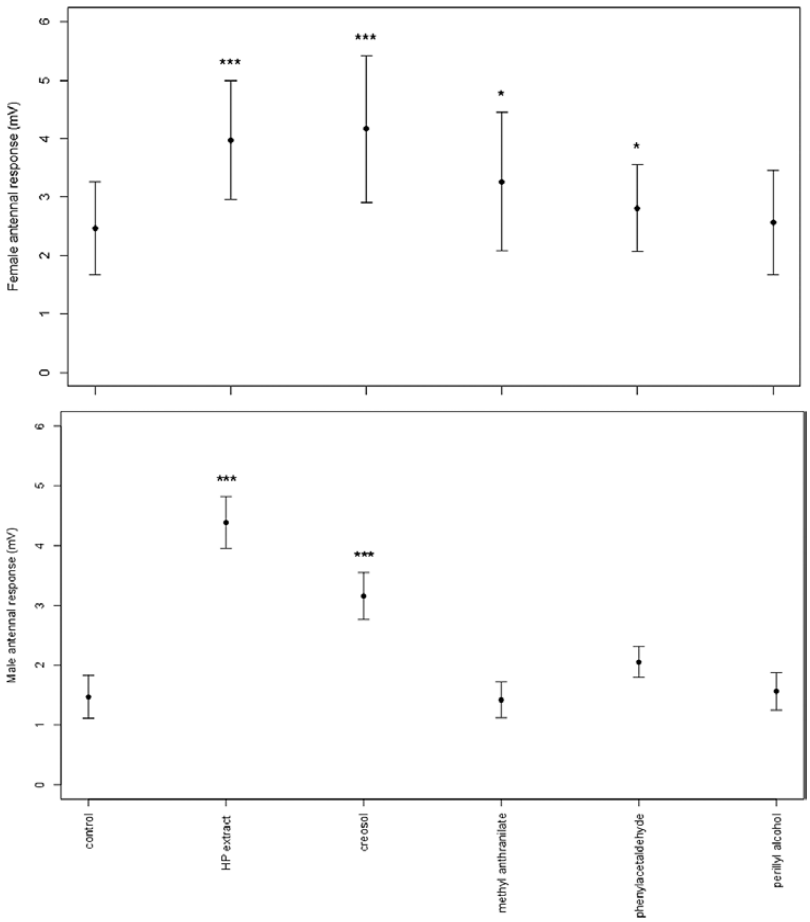

Fig. 3. Mean of female and male antennal response (mV) ± SEM to hair pencil extracts and four aromatic compounds identified in male hair pencils of the Ccoffee berry moth. Treatments labeled are significantly different than the antennal response to the control. ***$P \leq 0.001$, **$P \leq 0.01$, *$P \leq 0.05$.

and methyl anthranilate (33%). Phenylacetaldehyde and 2-phenyl ethanol were already identified as an attractant in numerous species of insect, including some Lepidoptera. Both molecules were identified in male HP of Noctuidae (Bestmann et al. 1977). According to Pherobase (El-Sayed 2016), creosol, perillyl alcohol, and methyl anthranilate are not components of male Lepidoptera HPs, neither of female pheromone. Creosol is a floral compound mainly identified in Asparagales and once reported as present in an Arthropod, although the role was not specified (Taira and Arakaki 2002). Methyl anthranilate is also a plant fragrance, and there are few records as an insect product. It was identified as a pheromone compound in ants (Duffield et al. 1980; Lloyd et al. 1984; Lloyd et al. 1989; Oldham et al. 1994a, b) and was previously identified as an attractant in the soy beetle (Imai et al. 1997), thrips (Murai et al. 2000), and beneficial insects (James 2005). Perillyl alcohol is a trickier molecule, mainly found in the essential oil of various plants such as lavender, sage, and known as a mosquito repellent and as a treatment for cancer. We did not find any report on this compound as an animal product.

The significant EAG responses on both male and female antennae induced by male HP extracts showed that both sexes detected the male emission (with no significant difference of antennal response between male and female), suggesting that the blend released may act on male and female behavior, qualifying it as a pheromone. Numerous studies show a correlation between EAG responses to a compound and a behavioral response, either positive or negative (Floyd et al. 1976, Baker and Haynes 1989, Landon et al. 1997, Liendo et al. 2005, Dötterl et al. 2006). Mating behavior did not occur under laboratory conditions; thus, we were not able determine the role of HP emission on male and female reproductive behavior.

Significant responses of creosol on female and male antennas compared to the control (with no significant difference of antennal response between male and female) suggest that this compound plays a behavioral role on both conspecific male and female CBM during courtship and/or the mating period. Significant responses of methyl anthranilate and phenylacetaldehyde on female antennas compared to the control only could also suggest a role on female behavior.

Behavioral bioassays on males and females with HP extracts and HP volatile compounds are necessary to understand the role of each molecule in CBM courtship behavior.

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