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SHORT COMMUNICATION

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Larval salivary glands are a source of primer and releaser pheromone in honey bee (*Apis mellifera* L.)

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Abstract A brood pheromone identified in honeybee larvae has primer and releaser pheromone effects on adult bees. Using gas chromatography–mass spectrometry (GC–MS) to evaluate fatty acid esters—the pheromonal compounds—in different parts of the larvae, we have localized the source of the esters as the larval salivary glands. A histochemical study describes the glands and confirms the presence of lipids in the glands. Epithelial cells of the gland likely secrete the fatty acids into the lumen of the gland. These results demonstrate the salivary glands to be a reservoir of esters, components of brood pheromone, in honeybee larvae.

Introduction

Social insects have evolved completely dependent on adults for survival. To provide optimal care to the larvae, nurses must recognize the various brood instars, their age, sex, and needs. To date, the honeybee *Apis mellifera* L. is the only social insect species in which a brood pheromone (BP) has been identified. A blend of ten fatty acid methyl and ethyl esters produced by larvae have been shown to possess releaser effects, like the capping of the cells (Le Conte et al. 1994), and the recognition of the larval age and needs (see Slessor et al. 2005). The esters also have primer effects stimulating hypopharyngeal glands of nurses (Mohammedi et al. 1996) or inhibiting ovary development of the workers (Mohammedi et al. 1998). The full blend of ten esters, brood pheromone, modulates the behavioral development of young bees and stimulates workers for pollen foraging (see Slessor et al. 2005).

Fatty acid esters could be biosynthesized in the fat body, cuticular glands, or in an unknown specialized gland. The importance of BP in the honeybee colony leads us to search for sites of production and secretion in larvae. In this work, we used GC–MS analysis to search for a putative gland or tissues in which a high concentration of BP would be found. Initial investigations focused on the external cuticular surfaces. Differences in the concentration of esters on the cuticle were used to locate a secreting site.

Our search for the compounds on the external surface of the larvae showed that the highest amounts of esters are found on the anterior part, around the head. By examining different tissues, we have found the highest concentration of BP in the salivary gland. A histochemical study of the salivary glands revealed a staining pattern consistent with secretion/storage of lipids.

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Materials and methods

General

Honeybee larvae were from a mixture of European subspecies, typically used in the southeast of France for beekeeping, primarily *mellifera*. Experiments were done with the fifth instar worker larvae taken from a comb when they were moving up in the cells. They were kept in an incubator at 34°C and 70% RH for 4–5 h after their removal from the colony. The esters were quantified by GC (Varian 3900, split splitless injector and flame ionization detector) with a HP-INNO-Wax capillary column (length 25 m, internal diameter 0.20 mm, film thickness 0.2 μm). The oven temperature was programmed from 60 to 180°C at 7°C/min, then to 210°C at 1°C/min, and to 230°C at 10°C/min, and held at 230°C for 5 min.

The identity of the esters was confirmed by gas chromatography–mass spectrometry GC–MS. The GC–MS system was a Polaris ion-trap mass spectrometer/Trace 2000 GC (ThermoQuest) equipped with a PTV injector and a Varian CP-Sil8CB MS capillary column (length 30 m, internal diameter 0.25 mm, film thickness 0.25 μm). The system was used in electron impact ionization mode at 70 eV. Three diagnostic ions for each compound allow identification for methyl esters, $M^{+\cdot}$, M-31, M-32 (loss of methanol), and $M^{+\cdot}$, M-45, M-46 (loss of ethanol) for ethyl esters.

Extracts were fractionated on a silica gel column (silica 60, particle size 40–63 μm) made of 0.71 g of silica in a Pasteur pipette. Extract (100 μl) was placed at the top of the column and eluted with 4 ml of isohexane. The elution solvent was changed to 2 ml of a mixture of 94% isohexane and 6% diethyl ether, which eluted the esters. A 250-μl aliquot of this eluent was concentrated under nitrogen flow to 2 to 5 μl with 1.5 μl injected onto GC. The same procedure was used for GC–MS injections.

The amount of each ester was quantified by GC utilizing the internal standard and a standard line obtained by injection of quantities of each of the esters: methyl and ethyl of palmitate oleate, stearate, linoleate and linolenate.

Cuticular rinses

Cuticular rinses were obtained by immersing the anterior (50% of the larval body length) or posterior portion of larvae in 500 μl solvent (98.5 isohexane: 1.5 diethyl ether) containing methyl heptadecanoate as an internal standard. One replicate consisted of ten larvae, individually immersed in the same aliquot of solvent for 1 minute. Six replicates were analyzed.

Glandular and tissue extracts

Having identified the larval anterior part as the potential pheromone source, we searched for possible glandular structures associated with the mouth of the larvae.

We first looked at a possible gland around the mouth: the first 3 mm of the anterior part of ten larvae was cut off, starting from the mouth and crushed in solvent (500 μl, 98.5 isohexane:1.5 diethyl ether) for 10 min for chemical analysis. Five replicates were done.

Larvae were dissected as follows: (1) salivary gland, (2) the digestive tract and (3) the rest of the body including fat body and the cuticle. The dissected parts were immersed in solvent (500 μl, 98.5 isohexane: 1.5 diethyl ether) for 10 min and then crushed with a glass pestle. The extract mixture was centrifuged at 4,000×*g* for 20 min at 4°C. The supernatant was collected and concentrated to 100 μl under a flow of nitrogen. For each replicate, the tissues of four larvae were pooled for extraction. Eleven replicates were done. Before dissection, five larvae and five salivary glands were weighed.

Hemolymph analysis

Esters could be produced in one tissue and then transported to a secretion site *via* the hemolymph (Jurenka et al. 2003). Thus, it was necessary to investigate whether esters are present in hemolymph. Hemolymph (30 μl) was removed from one larva by suction. Briefly, a microcapillary tube was attached to an opening made on the posterior dorsal cuticle of the larvae. Three separate samples of hemolymph, pooled from 4, 10, or 40 larvae, were extracted with 200 μl solvent (98.5 isohexane: 1.5 diethyl ether). We used increased numbers in case of a concentration effect.

Carrier proteins may not necessarily release their ligands during an extraction, so we did two further experiments. First, the hemolymph from ten larvae (500 μl) was treated with trypsin to digest a potential carrier protein. Trypsin (Sigma, from porcine pancreas, 1,000–1,500 BAEE units/mg) was added to the hemolymph at a concentration of 0.25% w/w. The mixture was vortexed, incubated at 37°C for 3 h, and the aqueous mixture was extracted with 200 μl of 98.5 isohexane: 1.5 diethyl ether. Second, methanol (300 μl), which precipitate proteins, was added to 300 μl of hemolymph (final concentration of methanol 50% v/v). The extract was centrifuged to extract the supernatant. Three replicates of each experiment were done.

The same GC and GC–MS protocol described for glandular extracts was used for hemolymph.

Histochemistry

Larvae of the fifth larval instar were removed from the comb with forceps and fixed either in FAA (25% formalin, absolute alcohol, glacial acetic acid, 1/1/8, V/V/V) for general histology or in the lipid-preserving Ciaccio's solution for specific detection of lipids. This fixative consisted of 5% potassium bichromate, 25% formalin, glacial acetic acid: 80/15/5: V/V/V (Martoja and Martoja-Pierson 1967). After 12 h fixation at 4°C, the specimens were rinsed in distilled water, dehydrated in alcohol series (70–100%) and processed to be sectioned. The FAA-fixed larvae were embedded in 2-hydroxyethyl methacrylate resin (Technovit 7,100 embedding kit, Heraeus-Kulzer) according to the manufacturer's instructions. Serial, longitudinal sagittal, 3-μm thick sections were cut using a retraction microtome and collected on microscope slides. They were stained to visualize insoluble polysaccharides according to the Periodic Acid/Schiff's reagent procedure and proteins by Naphthol Blue Black (see El Maâtaoui and Pichot C1999). The specimens destined to lipid detection were embedded in paraffin and transversally sectioned at 6–7 μm thickness using a rotary microtome and stained with Sudan Black B (SBB) to visualize neutral and acidic lipids. SBB is a diazoic dye having a very elevated solubility coefficient in the acidic and neutral lipids that are stained black. More than ten larvae were sectioned (five imbedded in resin and five in paraffin) and observed by descriptive analysis.

Results

Cuticular rinses

No traces of esters were detected on the posterior part of the larvae. They were detected only in cuticular rinses of the anterior section of the larvae (Fig. 1). We found similar composition of esters compared to previous comparable analysis (Trouiller et al. 1991).

Glandular and tissue extracts

Sections of the larval mouthparts of the head contained traces of the esters. We would expect a high level if a gland was contained in this part. This suggests that ester secretion occurs at or near the mouthparts.

The digestive tract and the salivary glands are the only two structures known to be connected to the mouth of the larvae. No esters were detected in extracts of the digestive tract. The salivary gland extracts contained significantly more ethyl and methyl esters than extracts from the rest of the larval body (Fig. 2). The total amount of esters per larva was significantly different in the salivary glands [321±87 ng (mean±SE)] compared to the rest of the larvae (87±23 ng) (t test t=3.32, df=20; $P<0.003$). The total amount of esters on the rest of the larval body was not significantly different (t test t=0.387, df=15; P=0.705) from cuticular rinses of the anterior part (107±28 ng) (Figs. 1 and 2).

No esters were detected in untreated hemolymph, trypsin digested hemolymph, or methanol precipitated proteins hemolymph.

The weight of the salivary glands represented 0.13% of the total larval weight. Thus, the concentration of esters is 1,964.06±340.94 ng/mg of tissue in the salivary gland and, in contrast, the concentration in the rest of the larva is 0.61±0.01 ng/mg of tissue. These data clearly demonstrate that the esters are concentrated in the larval salivary gland.

Histochemistry

The salivary gland is a well-developed structure occupying a large space in the ventral part of the larvae (Fig. 3a). It is a highly convoluted tubular gland extending from the anterior to the posterior parts of the body. A tubular structure, connected to the ventral part of the mouth, divides into two posterior secretory glands. Histochemical tests revealed SBB-positive material in the lumen of the gland, indicating the presence of lipidic substances (Fig. 3b,c). Structurally, the salivary gland can be divided into the anterior and posterior parts. The anterior part is composed of very thin epithelium with no secretory vesicles, which suggests a conduction function. The posterior part, which divides into two parts, exhibits a cuboidal epithelium rich in lipid vesicles (Fig. 3c). The cells that compose the epithelium show an apical surface opening on the gland lumen and a basal surface laid on a thin basement membrane that is linked with the surrounding fat body (Fig. 3a,b). These cytological features are characteristic of secretory cells (Martoja and Martoja-Pierson 1967).

Discussion and conclusion

The present study demonstrates that pheromonal esters might be secreted on the anterior part of the larvae, around the mouth area. Dissection of larvae has revealed that the salivary gland is the reservoir for the brood pheromone esters. These esters have already been found in salivary glands of other adult insects (Valterová et al. 2001), but they were not found in the larval salivary glands of insects before. The amounts of the esters of the larvae is the most important at the end of the fifth instar (Trouiller et al. 1991), just before the capping of the cells by the workers and the silk production by the larvae, providing adult food provisioning to the larvae (see Slessor et al. 2005). Whether the larvae include the pheromone into the silk of the cocoon to use it, as a chemical releaser, is a very interesting issue that we are exploring.

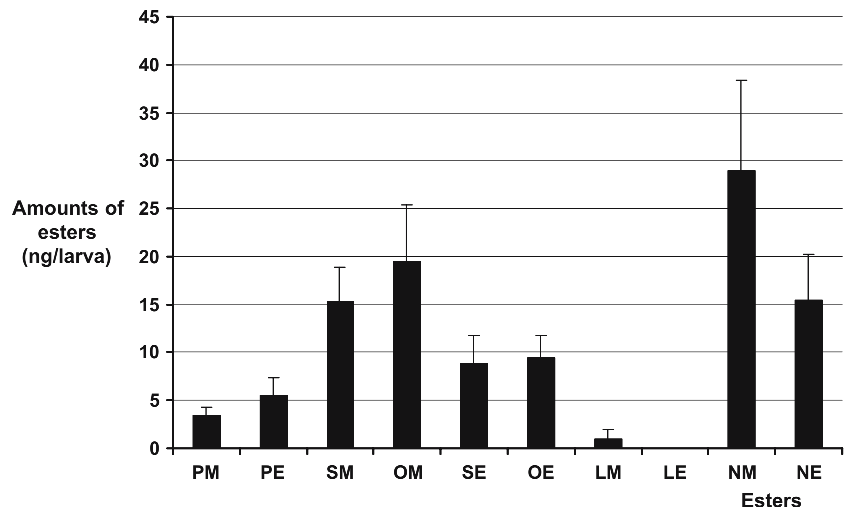

Fig. 1 Amounts of methyl and ethyl esters (components of brood pheromone) in rinses of the anterior part of the honeybee body of ten pooled larvae (mean± SE; n=6). *PM* Methyl palmitate, *PE* ethyl palmitate, *OM* methyl oleate, *OE* ethyl oleate, *SM* methyl stearate, *SE* ethyl stearate, *LM* methyl linoleate, *LE* ethyl linoleate, *NM* methyl linolenate, *NE* ethyl linolenate

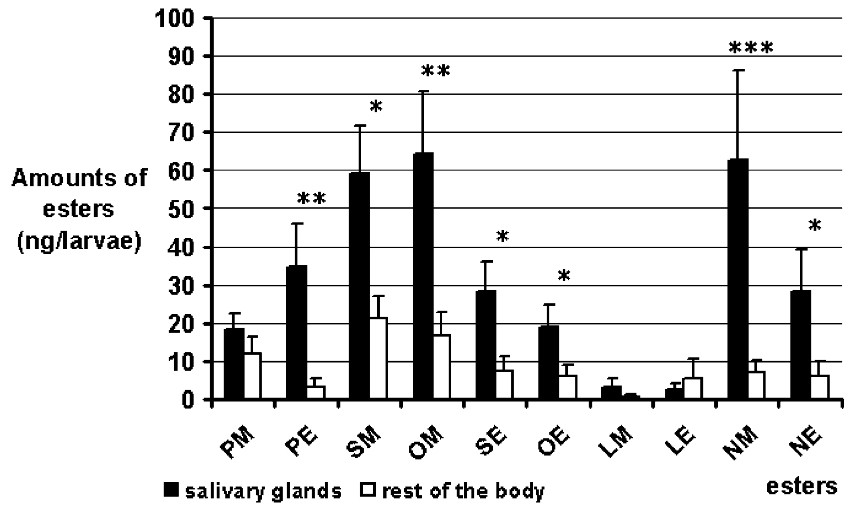

Fig. 2 Amounts of methyl and ethyl esters in the salivary glands, compared to the amount of each ester in the rest of the larval body. Parts of four larvae were pooled in each replicates (n=11) and amounts are expressed per larva. Pairs of bars marked with a *single asterisk*, *double asterisks*, or *triple asterisks* are significantly different between the salivary gland and the rest of the body, with $P<0.05$, 0.01, or 0.001, respectively. For compound names, see Fig. 1

Our histological study showed that the salivary glands are a tubular, threadlike structure that spreads throughout the length of the honeybee larva, as already shown (Anglas 1901). Microscopic observations have revealed a secretory function of the cells composing the epithelium of the posterior part of the salivary gland. This finding would be

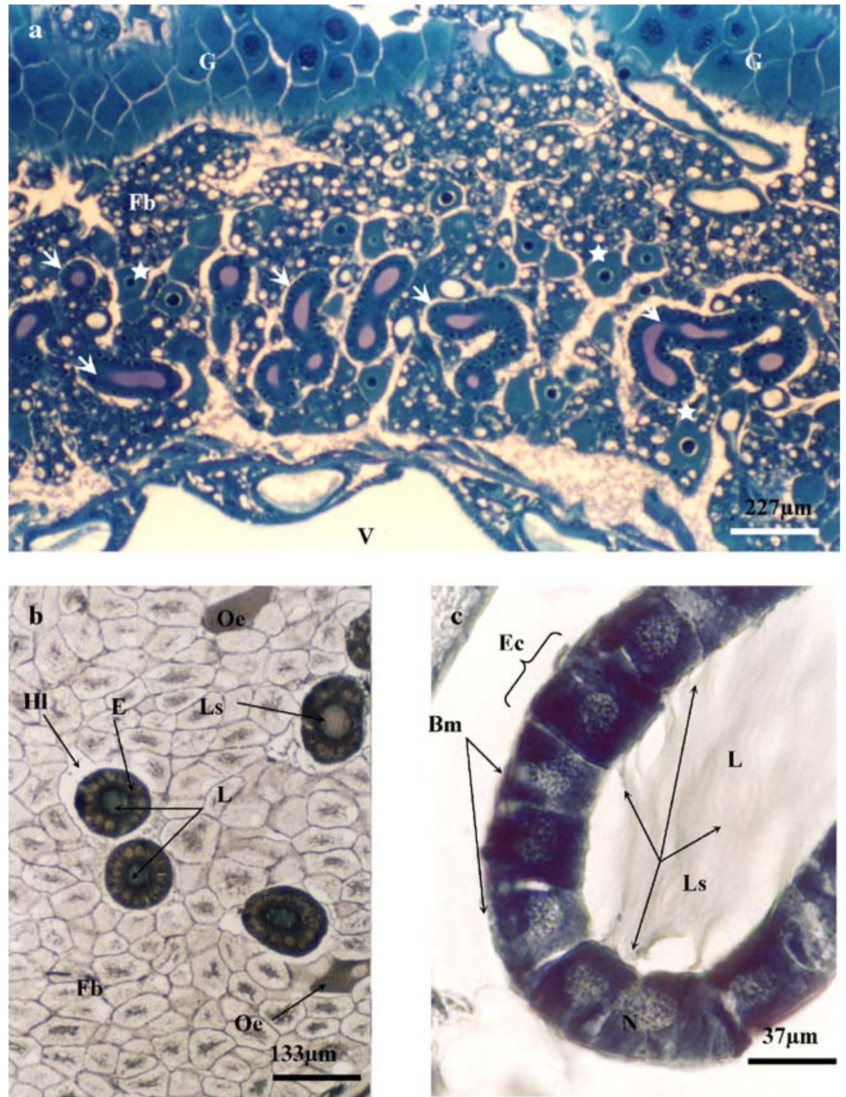

Fig. 3 Histochemistry of the posterior part of the salivary glands. **a** Longitudinal section showing the convoluted, tubular structure that traverses the salivary glands (*arrowheads*) in the ventral part of the larva (V). *G* midgut; *stars* oenocytes. **b** Salivary glands of L5 instar larva cut transversally, Sudan black B stained. Epithelium (E) as well as lumen secretions (Ls) are lipid positive. Tissues surrounding the gland are part of the fat body (Fb). **c** Salivary glands of L5 instar larva cut longitudinally, Sudan black B stained. Secretory epithelial cells (Ec) are clearly visible. These cells and their secretions (Ls) are lipid-positive. *L* lumen, *Fb* fat body, *E* epithelium, *N* nucleus, *Bm* basement membrane, *Ec* secretory epithelial cells, *Oe* oenocyte, *Ls* lipidic secretions, *Hl* hemolymph

consistent with the salivary glands being the source of the fatty acid esters. The salivary glands in honeybee larvae have been known for their function in silk secretion, necessary for the construction of a cocoon preceding the nymph instar (Anglas 1901, Silva-Zacarin et al. 2003). Our results indicate that those glands have at least one additional function, that is, the release of a brood pheromone composed of ten fatty acid esters. The absence of esters in the hemolymph suggests that the esters are not being transported through the hemolymph from some other tissue (e.g., the fat body). The presence of lipid vesicles, in the epithelium of the posterior portion of the salivary glands, suggests that the salivary glands might synthesize pheromonal esters, but further investigations using labeled precursors must be carried out to establish this synthetic function.

To conclude, this study shows that the salivary glands of the larvae can be considered the reservoir for the fatty acid esters that constitute brood pheromone.

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References

Anglas J (1901) Observations sur les métamorphoses internes de la guêpe et de l'abeille. Bull Sci Fr Belg 34:363–473

El Maâtaoui M, Pichot C (1999) Nuclear and cell fusion cause polyploidy in the megagametophyte of common cypress, *Cupressus sempervirens* L Planta 208:343–351

Jurenka RA, Subchev M, Abad JL, Choi MY, Fabrias G (2003) Sex pheromone biosynthetic pathway for disparlure in the gypsy moth, Lymantria dispar. Proc Natl Acad Sci U S A 100(3):809-814

Le Conte Y, Sreng L, Trouiller J (1994) The recognition of larvae by worker honeybees. Naturwissenschaften 81:462–465

Martoja R, Martoja-Pierson M (1967) Initiation aux techniques de l'histologie animale. Masson et Cie, Paris, pp 184–200

Mohammedi A, Crauser D, Paris A, Le Conte Y (1996) Effect of a brood pheromone on honeybee hypopharyngeal glands. C R Acad Sci III 319(7):769–772

Mohammedi A, Paris A, Crauser D, Le Conte Y (1998) Effect of aliphatic esters on ovary development of queenless bees (*Apis mellifera* L.). Naturwissenschaften 85(9):455–458

Trouiller J, Arnold G, Le Conte Y, Masson C (1991). Temporal pheromonal and kairomonal secretion in the brood of honeybees. Naturwissenschaften 78:368–370

Silva-Zacarin ECM, Silva de Moares RL, Taboga SR (2003) Silk formation in the larval salivary glands of *Apis mellifera* (Hymenoptera: Apidae). J Biosci 28:753–764

Slessor KN, Winston ML, Le Conte Y (2005) Pheromone communication in the honeybee (*Apis mellifera* L.). J Chem Ecol 31:2731–2745

Valterová I, Urbanová K, Hovorka O, Kindl J (2001). Composition of the labial gland secretion of the bumblebee males *Bombus pomorum*. Z Naturforsch 56c:430–443