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Using a proteometabolomic approach to investigate the role of Dufour's gland in pheromone biosynthesis in the social wasp *Polybia paulista*

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ABSTRACT

Dufour's gland is associated with the venom apparatuses of social wasps and bees. This location and its evolutionary adaptations indicate that it could be involved in the production of alarm pheromones in the social wasp *Polybia paulista*. To investigate this hypothesis, the volatile composition of this gland was analyzed and compared to that in the venom. Eighteen compounds were identified as secreted by Dufour's gland, and 16 of these compounds were also identified in the venom, suggesting that the compounds produced by the gland are secreted and mixed with venom in the venom reservoir of this wasp. These compounds were subjected to a field bioassay to investigate their potential action as alarm pheromones. Alcohols and aldehydes elicited the alert behavior in workers, luring them outside the nest, whereas acids attracted the workers in the direction of the source; fatty acid methyl esters elicited aggression. These results suggest that Dufour's gland produces alarm pheromones. To corroborate this hypothesis the proteomic complement of this gland was assigned using a shot-gun strategy; 59 proteins were identified, and the results indicate specialization of Dufour's gland for the metabolism of fatty acids (elongation, esterification unsaturation, reduction, and decarboxylation) in the biosynthesis of alarm pheromones.

Biological significance: The present knowledge about the role of Dufour's gland among aculeate Hymenoptera insects suggests that it may have many different roles related to the biosynthesis and secretion of chemical markers for different biological functions, though none are related to the elicitation of alarm behaviors for coordinating a mass attack of the colony against intruders. The present study combined the analysis of secreted volatile compounds (as metabolites) with proteome assignments and a field bioassay with synthetic compounds to clearly demonstrate that Dufour's gland does in fact biosynthesize alarm pheromones in social wasps. This strategy may be reproduced in other investigations related to pheromone production in other insects.

1. Introduction

Colonies of social insects (wasps, bees, ants and termites) contain large amounts of immature brood forms and nectar dew among other types of forage reserves, which are very attractive as food for different predators [1]. To protect the colony from attack by predators, social insects have developed a series of strategies for communicating danger to their nest-mates [2,3]. The chemical communication of a danger within the vicinity of the colony is an important defensive strategy used by large colonies of honeybees and social wasps [2], whereby alarm pheromones are released together with venom during stinging attacks [4,5]. The release of alarm pheromones is also important for coordinating colony defense, including a massive attack against the predator [1]. In addition to be used in the defensive strategies, pheromones also play important roles in the control of social activities and communication among the social insects; these compounds maybe used to regulate many of the intra/extra-colonial activities of this arthropod group [6,7]. Pheromones are produced by a large number of exocrine glands that secrete them as volatiles compounds [6,7]. These compounds may signal sources of food for foraging, regulate access to the colony, determine caste-specific behaviors or inter-caste interactions, and elicit/coordinate defensive behaviors of the colony [8,9].

Dufour's gland is anatomically well developed in social insects, and its diverse biosynthetic capabilities contribute to the many different functions attributed to it. In social wasps, chemicals secreted by the gland appear to be involved in nest-mate recognition [10], egg marking [11], larval rearing, and even nest defense [12]. The alarm is initially communicated through a series of individual behaviors by guard workers, which perceive the presence of an intruder within the nest vicinity and may produce a pulsed vibration from wing buzzing and/or scrape the nest surface with their mandibulae to alert nest-mates inside the hive of a potentially dangerous situation [13]. Chemical

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communication appears to be the second level of colony defense; indeed, the use of alarm pheromones is relatively common among social insects, which build large and complex colonies [2].

It has been demonstrated experimentally that alarm pheromones are released during the stinging action [4,5], and there are even some reports that alarm pheromones may be released into the air by defensive workers (guards) prior to an attack [6,7]. The release of alarm pheromones during an attack and/or at the site of wasp stinging serves to attract more workers to defend the colony in a coordinated action. In general, alarm pheromones are stored in the venom reservoir, together with toxins. Considering that both Dufour's gland and the venom gland are anatomically connected to the venom reservoir, the function of producing alarm pheromones to be used in the chemical communication of a danger to the colony has been attributed to this gland [14,15]. Such chemical communication can also be used to recruit nest-mates outside the nest for a coordinated attack against an intruder in the area of security around the nest [2]. Despite this evidence, the true function of Dufour's gland has been the subject of much speculation. Because this gland may have different roles in different groups of hymenopteran insects with different degrees of eusociality, we decided to investigate the involvement of Dufour's gland in the biosynthesis of alarm pheromones in the social wasp *Polybia paulista*. The gland in this species is well developed, and the colonies are large and complex, with alarm pheromones playing a very important defensive role. We evaluated the chemical profile of volatiles both from the venom and secretion from Dufour's gland and analyzed the proteomic complement of the gland to identify enzymes involved in the biosynthesis of alarm pheromones.

2. Materials and methods

2.1. Chemicals

All reagents were purchased from Sigma-Aldrich (Milan, Italy). A linear alkane series kit (C7–C34; Sigma-Aldrich) and fatty acids (C8:0–C12:0, C13:0–C17:1 and C18:0–C20:5; Sigma-Aldrich) were used to optimize the GC × GC–MS method and to build a GC × GC hydrocarbon library. Hexane was purchased from TEDIA (95% purity).

2.2. Dufour's gland samples

Workers of the social wasp *P. paulista* (Hymenoptera, Vespidae) were captured with a net from a geo-referenced nest at the São Paulo State University (UNESP) campus in Rio Claro, SP, Southeast Brazil (22°23′42.5″S 47°32′33.3″W) and were immediately frozen and dissected. Dufour's gland was carefully punctured and extracted in a pre-treated glass tube containing 300 μL hexane for 2 min at 28 °C with shaking. The extracts were centrifuged at 650 ×*g* for 5 min at 25 °C. The supernatants were collected and immediately analyzed by GC–MS.

2.3. Glassware and plastic pre-treatment

Considering the sensitivity of the GC–MS system, it was necessary to use glassware and other materials that were completely clean and without contaminants (e.g., plasticizers, mold release agents or manufacturing waste). The materials were washed with hexane (95% purity — TEDIA) for 60 min and then with a 0.5% (*w*/*v*) sodium hydroxide solution (SYNTH) for 60 min; the material was then washed again with hexane for 60 min. To check for possible contaminants, GC–MS analysis was performed on the solvents used in the various steps of glassware and plastic washing before the samples were analyzed.

2.4. GC/MS analysis

GG/MS analysis was carried out using a Shimadzu GC–MS quadrupole mass spectrometer system (mod. GCMS-QP2010 Ultra, Japan) with an RTx-5MS column (Restek, 5% phenyl–95% polidimethylsiloxane; 30 m × 0.25 mm DI, 0.25 μm). The capillary column was temperature-programmed as follows. The column temperature ranged from 80 °C to 270 °C in a non-linear gradient of 60 min. The initial temperature was adjusted to 80 °C, held for 2 min, gradually increased at 8 °C/min to 130 °C, held for 2 min, increased at 8 °C/min to 200 °C, held for 8 min, increased at 10 °C/min to 270 °C, and finally held for 26 min. The solvent delay was 5 min, and the equilibrium time was 3 min; the injection volume was 1 μL in splitless (50 s) mode, and the injector temperature was set at 250 °C for the entire run. The carrier gas was helium (purity 99.995%) delivered at a constant flow rate of 9 mL/min at an initial pressure of 102.1 kPa. The sample was analyzed in full-scan mode at a scan speed of 1000 amu/s and a sampling frequency of 1 spectra/s over a mass range of 30–500 m/z. The interface and ion source temperatures were 280 °C and 280 °C, respectively. The MS ionization mode parameters were as follows: electron ionization (EI), a filament bias of −70 eV, and a detector voltage of 0.98 kV.

2.5. Data analysis

GC–MS solution software was used for instrument control and data processing. The National Institute of Standards and Technology (NIST) mass spectral library (version 11.0) and Shimadzu GC/MS Metabolites Spectral Database library were used for compound identification of each peak.

2.6. Analysis of standard hydrocarbons

The criteria used for reliable identification of each compound consisted of the following: i) a mass spectral match factor (S) of the deconvoluted mass spectra based on the NIST 11 library and Shimadzu GC/MS Metabolites Spectral Database Library higher than 870 (for experimental data presenting a signal-to-noise ratio ≥120) and ii) linear retention index differences ($I = LRI_{exp} - LRI_{lit} \leq \pm 25$ index units), where RI*exp* is the retention index calculated for the first dimension of the GC–MS analysis and RI*lit* is the retention index reported in the literature for the RTx-5MS GC column or an equivalent column.

2.7. Shotgun proteomics

In-solution digestion was used for the shotgun strategy. For this purpose, the proteins (100 μg) from Dufour's gland extracts were solubilized in 50 mM ammonium bicarbonate, pH 7.9, containing 7.5 M urea for 60 min at 37 °C to denature the proteins, which were then reduced with 10 mM DTT at 37 °C for 60 min. After this treatment, the proteins were alkylated with 40 mM iodoacetamide at 25 °C for 60 min in the dark. The samples were diluted five-fold with 100 mM ammonium bicarbonate, pH 7.8, and 1 M calcium chloride was added to the samples to a final concentration of 1 mM. Non-autolytic trypsin (Promega) was added to the denatured protein solution (1:50 trypsin:protein, *w*/w) and incubated for 18 h at 37 °C. The samples were flash frozen in liquid nitrogen to quench the enzymatic digestion. The digested samples were desalted using an SPE C18 column (Discovery DSC-18, SUPELCO, Bellefonte, PA, USA) conditioned with MeOH, rinsed with 1 mL 0.1% TFA and washed with 4 mL of 0.1% (*v*/v) TFA/5% (*v*/v) ACN. Peptides were eluted from the SPE column with 1 mL of 0.1% TFA/80% ACN and concentrated to dryness using a Speed-Vac. The digested samples were stored at −80 °C until needed for analysis; the tryptic peptides were solubilized in 50% ACN and subjected to nanoLC-ESI-CID analysis.

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