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Behavioral and Olfactory Antennal Responses of *Solenopsis geminata* (Fabricius) (Hymenoptera: Formicidae) Workers to their Dufour Gland Secretion

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Respuestas Comportamentales y Olfatorias de Obreras de *Solenopsis geminata* (Fabricius) (Hymenoptera: Formicidae) a sus Secreciones de la Glándula de Dufour

RESUMEN - Se efectuaron pruebas comportamentales y electrofisiológicas para evaluar las respuestas de obreras de *Solenopsis geminata* (Fabricius) de diferentes tamaños a extractos de la glándula de Dufour. Se usaron medidas del ancho de la cabeza para determinar el tamaño de las obreras. Se evualuó la respuesta de seguimiento de ruta de obreras de diferentes tamaños a extractos de la glándula de Dufour proveniente de obreras de diferentes tamaños. Para cada grupo de obrera de determinado tamaño se le determinó la respuesta olfatoria por medio de electroantenografía (EAG). Se utilizó Cromatografía de Gases acoplada a Espectrometría de Masas (GC-EM) para determinar el perfil cromatográfico de la secreción de la glándula de Dufour para cada grupo de obreras. Las medidas morfométricas permitieron clasificar a las obreras de acuerdo a su tamaño en grandes, medianas y pequeñas. Las obreras medianas de *S. geminata* exhibieron una respuesta comportamental alta a los extractos glandulares de hormigas medianas. Las obreras medianas mostraron también una alta respuesta antenal a los extractos de obreras medianas. El perfil cromatográfico de la secreción de la glándula de Dufour producida por obreras muestra que cada tamaño exhibió un perfil característico de los tres componentes potenciales como feromona de ruta. Concluimos que las obreras de tamaño mediano exhibieron altas respuestas tanto comportamentales como olfatorias a la secreción de la glándula de Dufour. Lo anterior y el hecho de que sean las más abundantes en el área de forrajeo sugieren que este grupo de obreras está especializado en actividades de forrajeo.

PALABRAS CLAVES: Comportamiento, seguimiento de ruta, electroantenografía

ABSTRACT - Behavioral and electrophysiological tests were performed to evaluate the responses of workers of the ant *Solenopsis geminata* (Fabricius) from different size categories to Dufour gland extracts. Morphometric measures based in head widths across eyes were used to determine worker sizes. Trail following response of different worker sizes to Dufour gland extract from workers of different sizes was assessed. For each worker size category olfactory responses to Dufour gland extracts were determined using electroantennography (EAG). Gas chromatography and mass spectrometry (GC-MS) were used to determine the chromatographic profile of Dufour gland secretion for each worker size. Morphometric measures permitted to classify the workers of *S. geminata* as large, medium and small workers. Medium *S. geminata* workers displayed a significantly higher behavioral response to Dufour gland extracts produced by medium size workers. Similarly, medium workers showed a significantly higher EAG response to Dufour gland extracts produced by medium sized workers. Chromatographic profile of Dufour gland secretions produced by workers showed that each size category exhibited a characteristic profile of the three main components considered as potential trail pheromone constituents. This work showed that medium workers of *S. geminata* exhibited a high trail-following behavior as well as a high antennal response to Dufour gland secretion. This and their relative abundance in field foraging areas, suggest that medium-sized workers are specialized in foraging activities.

KEY WORDS: Trail-following behavior, electroantennography

Fire ants constitute a large group of species belonging to the genus *Solenopsis* and native to the New World; many of them are of considerable economic importance due to their action as potential biological control agents or as pests (Smith 1936, Travis 1941, Risch & Carroll 1982, Way *et al.* 2002). They exhibit an efficient foraging behavior which involves a sophisticated system of communication to orientate and recruit other members of the colony towards food sources and to find new nesting sites (Wilson 1959, 1962; Bell & Cardé 1984). Communication is performed utilizing a trail pheromone produced in the Dufour gland of workers, which has been studied at both chemical and behavioral levels in various species of *Solenopsis*, including *S. invicta* (Buren), *S. xyloni* (McCook), *S. geminata* (Fabricius) and *S. richteri* (Forel) (Barlin *et al.* 1976, Vander Meer *et al.* 1988). Nevertheless, despite the interesting attempt by Brand *et al.* (1973), a clear relationship level of perception and responsiveness to trail substances and worker subcastes, has not been investigated so far. Moreover, field observations (Brindis *et al.* personal observation) suggest that the medium size classes of *S. geminata* display some more differentiated activities related with foraging and food provisioning compared to minor and large workers. The objectives of this study are: 1) Evaluate an eventual difference in behavioral and olfactory responses to the Dufour gland secretion in function of the releaser and receptive worker size, and 2) Establish the existence of a relationship between the chemical profile of the Dufour gland secretions and the different worker sizes.

Material and Methods

Ants. Workers of *S. geminata* were collected in the foraging area of 13 nests in the municipality of Cacahoatán (14° 59´ N, 92° 10´ W), Chiapas, Mexico. They were transported to the laboratory and maintained in plastic containers under a relative humidity of 70 ± 5% and a temperature of 25 ± 3°C. Food provided every 24h consisted of a 10% sugar solution soaked onto cotton wool.

Morphometric measures for worker size determination. Head width across eyes measures of collected workers were recorded using a binocular microscope (Nikon mod SMZ-U 1:10) with a micrometric ruler. Once the morphometric measurements for different worker sizes in the foraging area were obtained, the number of workers in each size class for the 10 remaining nests was counted in order to establish the distribution of the different worker sizes in the foraging trail.

Sample preparation. The worker ants were immobilized at -20°C for a period of 5 minutes. The Dufour gland was dissected and separated from the poison gland using a stereomicroscope and a petri dish containing 10 ml of distilled water. Ten Dufour glands were placed in a glass tube containing 700 µl of hexane (HPLC grade) for five minutes, after that the glands were withdrawn and the extract concentrated with nitrogen to 100 µl, thus resulting in 10 µl for each gland. The extracts were stored at -20°C for later use in bioassays and electroantennography.

Worker behavioral response to Dufour gland secretion. In order to assess worker response to Dufour gland extracts, the protocol described by Pasteels & Verhaeghe (1974) was followed with some minor modifications. A 10 cm diameter circle with 1 cm divisions was drawn onto a piece of filter paper (Whatman No. 1, Maidstone, England) and either 10 µl of extract or hexane (control) were applied along the circumference using a normal graphic pen (Blundell Harbin, Weymouth, England) allowing the solvent to evaporate over 20 seconds. The filter paper was then placed in a circular plastic container (20 cm diameter X 5 cm high). Finally a worker was placed in the centre of the plastic container. The number of divisions over which the ant traveled continually during 5 min was recorded. After each period of 5 min, which was considered as a repetition, both the filter paper and the worker were replaced. For each sample nest, the response of the workers of the different sizes was assessed for extracts from each worker size category. Fifty replicates were carried out for each randomly selected combination. The bioassays were performed the day after the workers were collected.

Electroantennography (EAG). For each worker subcaste, olfactory response to the Dufour gland extracts from the different subcastes previously defined was determined using the electroantennography (EAG) setup described in Malo *et al.* (2004). In order to assess the EAG response of the three different sizes of workers to the series of Dufour gland extracts, 10 µl of extract (one Dufour gland equivalent) were placed on a piece of filter paper (Whatman no. 1, Maidstone, England) and exposed to the air for 20 seconds, allowing the solvent to evaporate. The sample was placed in a Pasteur glass pipette for 40 seconds before being applied. New pipettes were prepared for each insect tested. The stimulus was applied at intervals of 2 min. The Dufour gland extracts of different sized workers were used for each EAG preparation and randomly selected. The control stimulus (hexane) was presented at the start and at the end of each EAG analysis. We used one antenna per worker in each size class and for each series of tested extracts. At least 20 workers were used for each size class.

Chemical analysis. The chemical analysis on the Dufour gland extracts was performed using a gas chromatography system Varian CP 3800 (Palo Alto, CA, USA) and a mass spectrometer Varian Saturn 4D 2200 (Palo Alto, CA, USA). The compounds were separated with a non-polar capillary column (DB-5 MS, 30 m x 0.25 µm internal diameter, J&W Scientific Folsom, CA, USA). The temperature program used started with an initial temperature of 50°C for 2 min increasing by 15°C per minute, reaching a final temperature of 280°C. The carrier gas was Helium at constant flow rate (1.0 ml/min). The mass spectra were obtained by electronic impact at 70 eV. Extracts (one Dufour gland equivalent) were injected using the "Splittles" mode. The chemical identification of the trail pheromone candidates was carried out by comparing the mass spectra of the natural compounds with published mass spectra (Vander Meer *et al.* 1981, Cruz-López 1994). The relative amount of the components was calculated from the sum of the areas of each one of the recorded peak. The extracts taken from the Dufour glands were analyzed for different worker size classes. Five replications from two different nests were carried out for each size class.

Statistical analysis. The statistical analyses were carried out using the statistical package STATISTICA for Windows, version 7.1, Statsoft, Inc. (2005). Data obtained from both the morphometric measurements (three nests) and chemical analysis (two nests) of the Dufour gland, were pooled because preliminary analysis showed that there was no significant difference between data. These data were analyzed by a principal component analysis. To determine which variables influence group separation, a correlation of the original variables and the principal components used were obtained. The behavioral responses of different size workers to the Dufour gland secretions were analyzed by means of a two-way ANOVA, whereby the first factor is extract origin with three levels (Dufour gland extracts of large, medium and small workers) and the second factor is tested worker subcaste (large, medium and small) assessed in the bioassays. The EAG amplitude values were analyzed using a two-way ANOVA where one factor is the assessed products (hexane, Dufour gland extracts of large, medium and small workers) and the second factor is tested worker subcaste (large, medium and small). When necessary, data were logarithmically or square root ($\sqrt{x}$) transformed to meet the assumptions of normality and homogeneity of variances. Those transformed data were obtained applying the Box-Cox family of transformations and transformation of ranks (Conover & Iman 1981). Significant ANOVAs were followed by posthoc Tukey test for multiple comparisons of means ($P \leq 0.05$).

Results

Worker size determination. The *S. geminata* workers display head widths across the eyes ranging from 0.05 mm to 0.15 mm. The workers that fell within the range 0.13 mm to 0.15 mm were classed as "large workers", those which were between 0.l0 mm and 0.12 mm "medium workers" and the remaining, between 0.05 mm and 0.07 mm, "small workers". The latter were statistically different from the two other subcastes ($F = 22133$; g.l = 2, 1497; $P \leq 0.05$) (Fig. 1A).

The number of workers counted within each size class in the 10 different nests showed that it was the medium sized workers who predominated in the foraging area ($F = 25.365$; df = 2, 27; $P < 0.05$). No difference occurred between the number of large and small workers present in the same area (Fig. 1B).

Trail following response of *S. geminata* workers to Dufour gland extracts. Behavioral test showed that the extract origin (from minor, medium or major workers) (nest 1: $F = 203.2$; df = 2, 441; $P < 0.001$), the worker size category of the tested ant (nest 1: $F = 1190.4$; df = 2, 441; $P < 0.001$), and the extract origin and tested worker size interaction (nest 1: $F = 225.6$; df = 4, 441; $P < 0.001$) significantly influenced trail-following responses of workers. Similar results were obtained for nest 2 (extract origin: $F = 189.2$; df = 2, 441; $P < 0.001$; tested worker size: $F = 1189.2$; df = 2, 441; $P < 0.001$); and the extract origin x tested worker size interaction ($F = 219.43$; df = 4, 441; $P < 0.001$).

In each combination, extracts from any worker size elicited higher responses than controls. The workers of the three size categories displayed a significantly higher trail-following response to Dufour gland extracts taken from ants of their own subcaste (Fig. 2A). However, the response to extracts taken from medium sized workers was always highest, independent of the tested worker subcaste (Fig. 2B). Similar results were obtained from nest 2. (data not shown)

Chromatographic profile of the Dufour gland secretions of *S. geminata* workers. Independent of the worker size category concerned, the secretion of the Dufour gland is composed of a mixture of alkaloids (venom components) and both saturated and unsaturated hydrocarbons (C_{13}–C_{25}) with an even and odd number of carbon atoms. In our study only compounds 1, 2 and 3 (Fig. 3) were considered as potential components of the trail pheromone of *S. geminata* as suggested by Barlin *et al*. (1976). Compound 1 has a mass spectrum characteristic of a bishomofarnesene with a M^+ 232 molecular ion. The mass spectrum of peaks 2 and 3 possess the M^+ 246 molecular ion displaying a fragmentation trishomofarnesene pattern.

When comparing the relative amount of the three components produced in the secretion of the different worker size, a positive correlation was observed between ant size category and relative amount of pheromone components

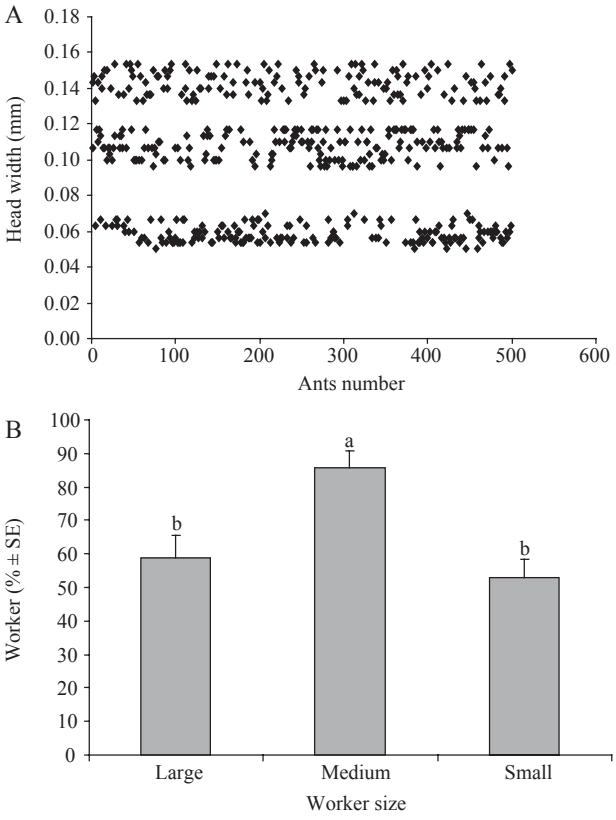

Fig. 1. A) Head widths of *S. geminata* workers collected in the foraging area. B) Worker sizes of *S. geminata* that predominated in foraging area. Different letters indicate significantly different comparisons (Tukey test $P \leq 0.05$).

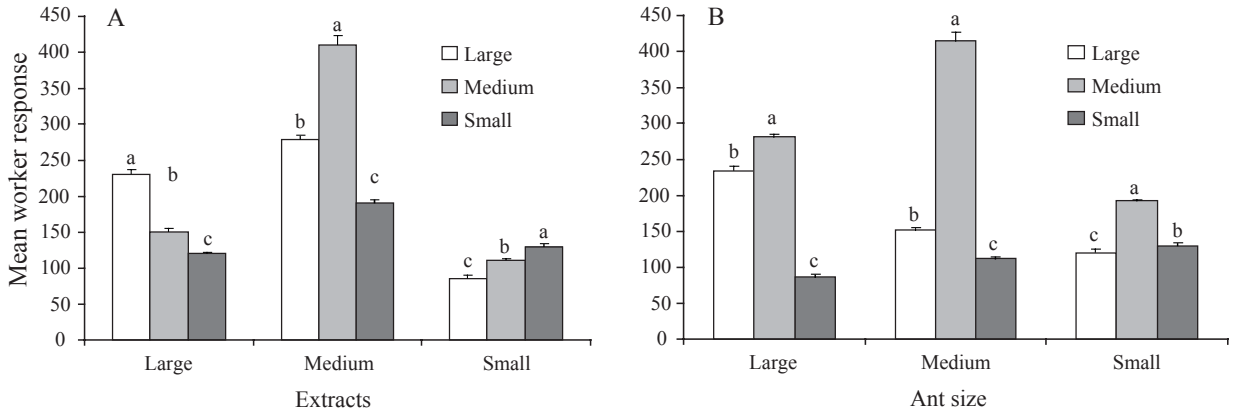

Fig. 2. Mean response of three sizes of *S. geminata* worker ants (n = 50 repetitions for each extract) to Dufour gland extract from different worker subcastes. Considering (A) extract origin; (B) tested worker subcaste (large, medium, small). Different letters indicate significantly different comparisons (Tukey test $P \leq 0.05$). n = 50 for each combination of extract and worker size.

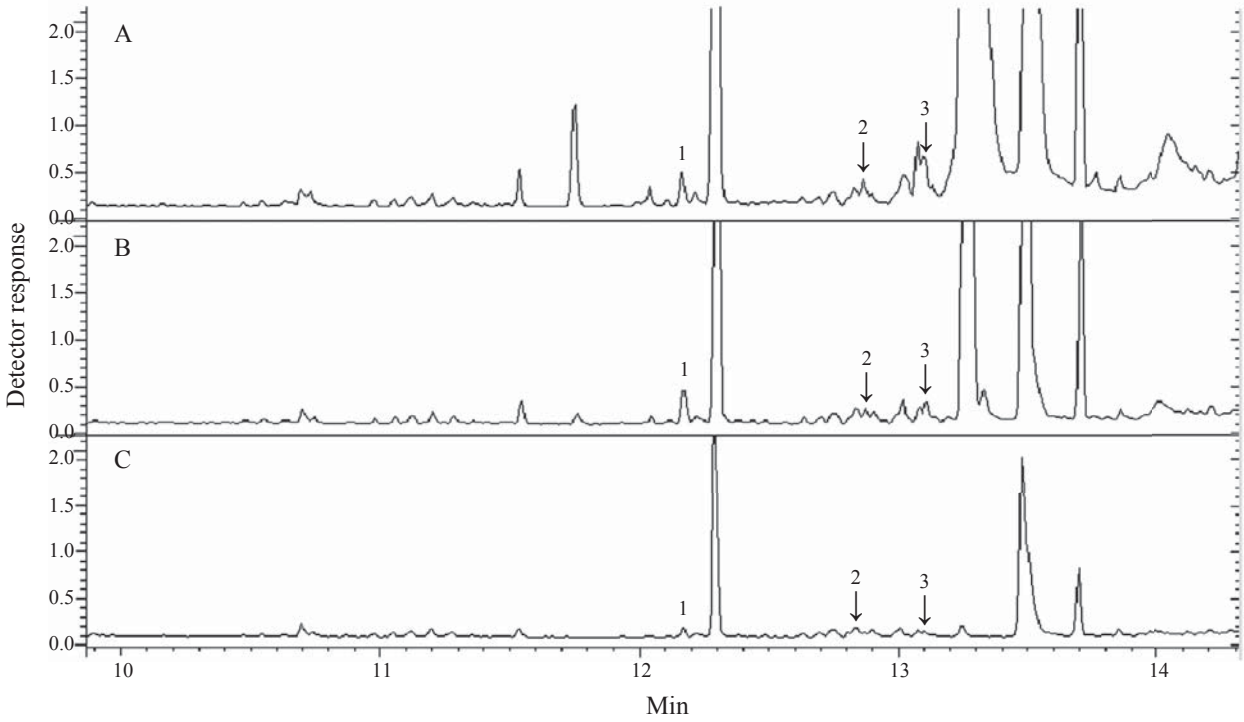

Fig. 3. Chromatographic profile of the Dufour gland in *S. geminata* workers (A) large workers; (B) medium workers; (C) small workers. n = 10 for each subcaste.

(F = 9.29; df = 4, 81; P < 0.05). Notwithstanding, the principal component analysis of the relative proportions of compounds 1, 2 and 3 shows that there is a natural grouping of these compounds according to the worker size (Fig. 4). The first two principal components explain 96.04% of the variance that exists in compounds 1, 2 or 3.

Electroantennography (EAG). The amplitude of the EAG responses obtained for each worker size to series of Dufour gland extracts showed that assessed products (F = 203.24; df = 3, 264; P < 0.05), worker subcaste (F = 145.; df = 2, 264; P < 0.05) and assessed products x worker size interaction (F = 121.82; df = 6, 264; P < 0.05) significantly influenced the antennal response in workers of the three subcastes (Fig. 5). The workers from each of the subcastes displayed a significantly higher antennal response to Dufour gland extracts produced by ants of their same size (Fig. 5A). Likewise, the workers showed a significantly higher response to Dufour gland extracts produced by medium sized workers rather than large and small workers (Fig. 5B).

The antennal response of the *S. geminata* workers to the different Dufour gland extracts was significantly different

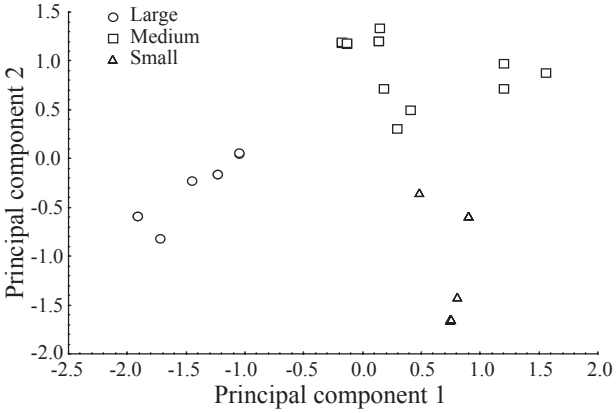

Fig. 4. Dispersion of principal components 1 y 2 corresponding to the three components considered in the CG-MS analysis of the Dufour glands in workers of *S. geminata*.

when compared with the response elicited with hexane (control). This antennal response varied depending on the subcaste of the worker being assessed and fluctuated within a range of 0.57 mV and 1.97mV.

Discussion

Bioassay results demonstrated that Dufour gland secretion of medium workers released a significantly higher trail following responses to all three subcastes compared with the Dufour gland secretion from the other two subcastes. We also registered that the intensity of the trail-following response in medium-sized *S. geminata* workers was significantly higher when compared with both major and minor workers. Of particular interest is the fact that medium sized workers of *S. geminata* show a greater antennal capacity in perceiving trail pheromones. This indicates that there is a direct relationship between trail-following and antennal responses between subcastes. Many other studies describe electrophysiological responses to pheromones (Kern & Bestmann 1993, Andryszak *et al.* 1990) but none consider electrophysiological responses between subcastes. Highly significant behavioral and antennal responses of medium workers, together with their relative abundance in the foraging area involved in food transport to the nest (Fig. 1 B and unpublished observations) may lead that medium-sized workers are specialized in foraging activities. In other polymorphic ant species a similar relationship has been observed between behavioral specialization and morphological subcaste. For example, in the harvester ant *Messor barbarus* (L.), the medium sized workers are more involved in food collecting activities (Heredia & Detrain 2000). Similarly, in *Acromyrmex lobicornis* (Emery) two morphological subgroups each with specific functions can be differentiated: minor workers which take care of the brood and fungus inside the nest and major workers which are usually found outside the nest providing food to the colony (Quirán *et al.* 2001). However, further behavioral studies are needed to understand the respective role of the different worker subcastes of *S. geminata* on foraging trails.

The efficacy of a secretion often depends on a mixture of substances, thus the relative proportion of the components may be critical. When comparing the chromatographic profiles of the Dufour gland secretions for *S. geminata* workers, a positive correlation was observed between ant size and secretion quantity. Interestingly, each worker subcaste show a characteristic profile of the three components considered as being essential in the trail pheromone (Barlin *et al.* 1976). This could indicate that the relative abundance of the substances is critical to act as an efficient trail pheromone. Therefore, it seems likely that there is a correlation between medium sized worker specialization and chemical composition of the secretion but not with pheromone quantity. Variation in the proportions of secretion components between castes of varying sizes have been reported previously. For example, in *S. geminata* Brand *et al.* (1973) found that variations in proportions of cis-2-methyl-6-n-undecylpiperidine (cis C_{11}) with trans-2-methyl-6-n-undecylpiperidine (trans C_{11}), both present in poison gland secretions, were greater in soldiers

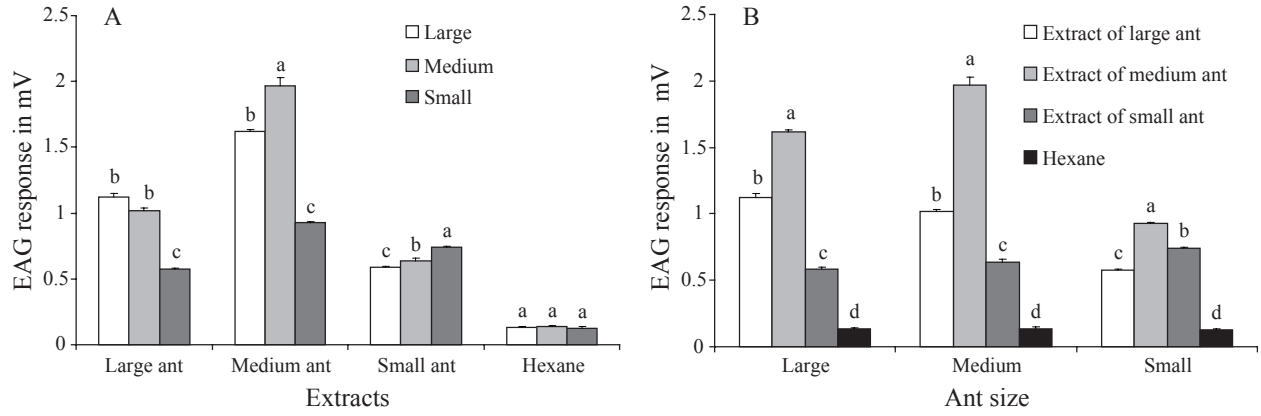

Fig. 5. Antennal response of three sizes of *S. geminata* workers to the equivalent of one Dufour gland secretion. Considering (A) assessed products (B) tested worker subcaste. Different letters indicate significantly different comparisons (Tukey test $P \leq 0.05$). n = 20 EAG records for each extract.

than in medium workers. Similarly for *S. invicta*, Deslippe & Guo (2000) reported that the venom alkaloid composition varied across worker size and age. Furthermore, in *Myrmica rubra* (L.) there was a variation in the proportion of 3-octanol and 3-octanone in worker mandibular glands across worker size (Tricot *et al.* 1972).

A final point of interest is that minor and major workers are less abundant than median workers in the trail foraging area but they show an antenal response and perception to the Dufour gland secretion; however it seems that they might play a different role to that of forager. In other polymorphic ants different functions have been observed between subcastes, for example in *A. capiguara* (Gonçalves), two worker subcastes were found on foraging trail, large forager were found transporting grass while minors patrolled the trail area for threats, playing a key role in alarm reaction (Hughes & Goulson 2001). We conclude that medium workers of *S. geminata* exhibited a high trail following as well as a high antennal responses to Dufour gland secretion. This and their relative abundance in the field foraging area suggest that medium-sized workers are specialized in foraging activities.

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