Short Communication

The Journal of Venomous Animals and Toxins including Tropical Diseases
ISSN 1678-9199 | 2012 | volume 18 | issue 4 | pages 441-445

Antibacterial properties of contact defensive secretions in neotropical *Crematogaster* ants

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Abstract: *Crematogaster* ants use their contact venoms to compete with other ants. Although those venoms are used primarily as repellent and toxic secretions, they may have other functions. The present study aimed to test the antibacterial property of abdominal venom of three neotropical *Crematogaster* ant species (*C. distans, C. pygmaea* and *C. rochai*) against gram-negative (*Escherichia coli* and *Pseudomonas aeruginosa*) and gram-positive (*Enterococcus faecalis* and *Staphylococcus aureus)* bacteria. Sterile filter paper was soaked with *C. distans, C. pygmaea* or *C. rochai* crude venom and placed on an agar dish that was inoculated with bacterial suspensions. The agar dish was incubated overnight at 37°C and examined for zones of growth inhibition. For each tested venom and bacterial strain, three venom concentrations were used, with six replicates for each concentration: 1, 2 and 4 DGE (Dufour's gland equivalent). The venom of *C. pygmaea*, but not those of *C. rochai* and *C. distans,* inhibited the growth of all tested gram-positive and gram-negative bacterial strains. This is the first evidence of antibacterial properties of contact venoms in *Crematogaster* ants and it supports the claim that ant venoms are multifunctional. It is hypothesized that only *C. pygmaea* venom showed antibacterial activities due to its nesting habits.

Key words: Hymenoptera, Formicidae, *Crematogaster*, sting apparatus, contact venoms.

Crematogaster Lund, 1831 is the fourth most speciose ant genus. Many species of this genus are dominant elements of tropical ant communities (1, 2). All of them possess the Formicidae sting apparatus in a derived state: while the most primitive ants use their sting to inject venom produced by the poison gland, *Crematogaster* species possess a spatulate sting utilized to apply contact defensive secretions from their hypertrophied Dufour's gland directly onto the integument of enemies (3, 4). This is facilitated by the unique arrangement of postpetiole and gaster, which allows the gaster and the sting to be pointed in nearly all directions (3, 4).

Chemical analysis of the Dufour's gland secretions of *Crematogaster* species from Europe, Papua New Guinea, Brazil and Kenya showed high diversity of defensive compounds including long-chain conjugated dienones, furanocembranoid diterpenes and trihydroxylated cyclohexane derivatives (5-10). *Crematogaster* contact venoms seem to be used primarily as repellent and toxic secretions in interference competition with other ants, as demonstrated in species from South America (*C. rochai* Forel, 1903, *C. distans* Mayr, 1879 and *C. pygmaea* Forel, 1904) and Europe [*C. scutellaris* (Olivier, 1792)] (11, 12).

However, *Crematogaster* venoms could have other functions, since multifunctional venoms seem to be the rule in ants (13). In *Solenopsis invicta* Buren, 1972 and *Pachycondyla goeldii* (Forel, 1912), for example, defensive and/or

offensive venoms are also used as antiseptic substances for nest and brood hygiene (14, 15).

The three species analyzed in the present study (*C. distans*, *C. pygmaea* and *C. rochai*) are common in northeastern Brazil. *Crematogaster pygmaea* is a soil-nesting species found in open sandy areas of the coastal zone, whereas *C. rochai* and *C. distans* are two sympatric arboreal species found in "caatinga", a savanna-like formation (16, 17). In *C. distans*, the main compound found in the Dufour's gland is a long-chain derivative (acetylenic tetraene) linked to a primary acetate [(13*E*,15*E*,18*Z*,20*Z*)-1-hydroxypentacosa-13,15,18,20-tetraen-11-yn-4-one 1-acetate], while in *C. rochai,* the main compounds are two furanocembranoid diterpenes [(1*R**,11*R**,12*R**)-6,19:11,12-bisepoxycembra-3,6,8(19),15-tetraene, and (1*R**,3*S**,4*S**)-3,4:6,19-bisepoxycembra-,6,8(19),11,15-tetraene] (7, 8).

In *C. pygmaea,* preliminary results obtained with thin layer chromatography showed that the Dufour's gland chemistry is similar to that of the European species *C. scutellaris*, in which a reaction takes place on the sting at the time of venom emission. Briefly, long-chain primary acetates [a series of C_{23} long-chain derivatives, with a (*E,E*)-cross-conjugated dienone linked to a primary acetate function] from the Dufour's gland are converted to highly electrophilic aldehydes by enzymes (an acetate esterase and an alcohol oxidase) from the poison gland (18). In *C. scutellaris*, and probably also in *C. pygmaea*, as indicated by the strong smell of acetic acid when ants are disturbed (especially when nests are excavated in the field), the acetic acid released via hydrolysis of the primary acetates acts as an alarm pheromone (18). In this study, we investigated the antibacterial properties of *C. distans*, *C. rochai* and *C. pygmaea* venom.

Colonies of *C. pygmaea* were collected on the campus of the State University of Ceará, in Fortaleza (WGS: 03°47'S 38°33'W), while those of *C. distans* and *C. rochai* were collected at Pentecoste (WGS: 03°48'S 39°21'W), about 70 km from Fortaleza. For each *Crematogaster* species, two colonies were maintained in the laboratory, in round plastic containers (23 cm high and 32 cm in diameter) with test tubes (*C. pygmaea*) or pieces of abandoned nests of *Nasutitermes* Dudley, 1890 (*C. distans* and *C. rochai*) as nesting places. Colonies were fed on sugar solution, water and freshly killed cockroaches.

Two gram-negative (*Escherichia coli* ATCC 25922 and *Pseudomonas aeruginosa* ATCC 27853) and two gram-positive (*Enterococcus faecalis* ATCC 29212 and *Staphylococcus aureus* ATCC 25923) strains were used to test the antibacterial property of the three venoms. Bacterial cultures were stored in tryptic soy agar (TSA – Difco – Becton, Dickinson and Company, France) at BOD (biochemical oxygen demand incubator) 23°C and later renewed to be used in the antibacterial assays.

Direct inoculums of bacterial suspensions were prepared to match the turbidity of a McFarland 0.5 standard. A sterile cotton swab was dipped into the suspension, pressed and rotated firmly against the side of the tube to remove excess inoculum from the swab, and swabbed evenly over the surface of a Mueller-Hinton agar plate (19). After inoculation, a sterile filter paper (2 x 2 mm) was soaked with *Crematogaster* crude venom and placed on an agar dish (one paper per dish), and incubated overnight at 37°C and examined for a zone of growth inhibition.

In order to transfer the venom of one *Crematogaster* worker to a filter paper, the latter was first seized with forceps. With another forceps, the ant was seized in the thorax region. This procedure induced the emission of venom on the sting, which was subsequently rubbed against the filter paper until no more venom was released. The total volume of venom secreted by one worker was assumed to represent the content of one Dufour's gland and was called a Dufour's gland equivalent (DGE). Different individuals were used when more than 1 DGE was transferred to the filter paper.

For each tested venom and bacterial strain, three venom concentrations were used, with six replicates for each concentration: 1, 2 and 4 DGE. In separate agar dishes, sterile papers (2 x 2 mm) without venom (one paper per dish; six replicates for each bacterial strain) were used as controls (24-hour incubation time).

The Kruskal-Wallis test was used to compare the mean inhibition zone diameters obtained at each DGE concentration, with the four bacterial strains, and the mean inhibition zone diameters obtained for each bacterial strain, with the three DGE concentrations. The frequency of positive replicates (i.e. with inhibition of bacterial growth) in treatment experiments, and the frequency of positive replicates in control experiments

(without venom) were compared using the Fisher exact test.

Our results showed that the venom of *C. pygmaea*, but not those of *C. rochai* and *C. distans*, inhibited the growth of all gram-positive and gram-negative bacterial strains tested. While 1 DGE was active (i.e. showed a significant difference between the frequency of treatment replicates with effect and the frequency of control replicates with effect) only on *S. aureus* (Fisher exact test, $p < 0.05$), 4 DGE resulted in 100% inhibition of all bacterial strains (Table 1). The inhibition zone diameter obtained for all bacterial strains tested with 2 or 4 DGE was nearly twice or three times that obtained with 1 DGE, and that obtained for *S. aureus* with 1, 2 or 4 DGE was always higher than the other three bacterial strains (Table 1). However, no significant difference was observed among the three venom concentrations, whatever the bacterial strain, nor among the four bacterial strains, regardless of the venom concentration (Kruskal-Wallis test, $p > 0.05$) (Table 1).

Most ants live in warm and humid environments that favor proliferation of pathogenic microorganisms. Furthermore, social characteristics of ant colonies such as high population density, relatedness of colony members and concentration of potentially contaminated food items make those insects a good medium for rapid spread of infectious diseases through the colony (20). To protect their colonies from pathogens, ants have developed effective defense mechanisms including the production of compounds with antibiotic properties (13).

One of the main sources of antiseptic substances in ants is the metapleural gland (21, 22). In *Crematogaster deformis* Roger, 1857, for example, the hypertrophied metapleural glands contain a mixture of phenols that possess antiseptic and repellent activities (23). The poison gland is another common source of antimicrobial substances in ants like *Solenopsis invicta*, *Pachycondyla goeldii* and *Myrmecia pilosula* Smith, F., 1858. In *S. invicta*, alkaloid venoms applied to eggs by the queen inhibit the growth of entomopathogenic fungi (14). In *P. goeldii*, the venom contains peptides (ponericines) with strong antimicrobial properties that may act as cleaning agents before prey is brought into the nest (15). In *M. pilosula*, peptides were found to present a broad-spectrum antimicrobial activity against gram-positive and gram-negative bacteria (24). Besides, peptides with antimicrobial activities are widespread in the venom of other arthropods, such as the social wasp *Agelaia pallipes pallipes* (Olivier, 1792), the wolf spiders

Table 1. Antibacterial activity of three concentrations of *Crematogaster pygmaea* crude venom. Data are reported as the mean ± SD (n = 6). The number of replicates that produced inhibition zone is indicated in parentheses

Bacteria	Inhibition zone diameter (mm)				
	1 DGE	2 DGE	4 DGE		Control
Gram-positive					
Staphylococcus aureus	4.8 ± 2.71 (5)**	8.5 ± 3.67 (6)**	7.2 ± 3.19 (6)**	NS	0
Enterococcus faecalis	1.5 ± 2.35 (2)^{NS}	4.3 ± 2.25 (5)**	4.3 ± 0.82 (6)**	NS	0
Gram-negative					
Escherichia coli	2.5 ± 2.81 (3)^{NS}	4.0 ± 2.37 (5)**	5.7 ± 1.37 (6)**	NS	0
Pseudomonas aeruginosa	2.7 ± 2.07 (4)^{NS}	4.5 ± 2.43 (5)**	5.3 ± 1.21 (6)**	NS	0
	NS	NS	NS		

DGE: Dufour's gland equivalent; NS: $p > 0.05$ – Kruskal-Wallis test; $^{**}p < 0.05$; ^{NS}: $p > 0.05$ – Fisher exact test.

Lycosa carolinensis Walckenaer, 1805 and *L. singoriensis* (Laxmann, 1770), and the scorpion *Pandinus imperator* (Koch, 1841) (25-28).

In the current study, evidence that contact venoms produced by *Crematogaster* ants possess antibacterial properties is presented for the first time. Interestingly, only the venom of *C. pygmaea* showed antibacterial activity. This fact could possibly be related to the chemical differences among venoms or/and different nesting habits. *Crematogaster pygmaea* is a ground-dwelling species that nests in humid and warm soils, possibly with higher exposure to pathogenic microorganisms than *C. distans* and *C. rochai*, which live in drier arboreal nests in semi-arid environments (2, 16, 17). Furthermore, *C. distans* and *C. rochai* are frequently found in *Nasutitermes* nests that could already be impoverished in microorganisms due to antimicrobial substances produced by the frontal glands of termite soldiers (17, 29).

In conclusion, the present results suggest that toxic repellent secretions produced by the hypertrophied Dufour's gland of *C. pygmaea* may also be used as antimicrobial agents for nest hygiene. The spatulate sting and the extreme gaster flexibility could be particularly suited to apply antimicrobial compounds on surfaces such as nest walls, prey, or brood cuticle.

ACKNOWLEDGMENTS

We thank Dr. Ana Maria S. Assreuy and Dr. Yves Roisin for reviewing a draft of the manuscript and improving the English text, as well as the three anonymous reviewers whose suggestions greatly improved our manuscript.

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SUBMISSION STATUS

Received: April 4, 2012.
Accepted: August 20, 2012.
Abstract published online: September 3, 2012.
Full paper published online: November 30, 2012.

CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

FINANCIAL SOURCE

Ana Heredia was supported by a fellowship (DCR-CNPq) and Yves Quinet by a grant from the National Council for Scientific and Technological Development (CNPq), project n. 473939/2004-5.

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