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A preliminary study on the antimicrobial activity of sting secretion and gastral glands of the acrobat ant *Crematogaster scutellaris*

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Abstract

The sting secretion of the common acrobat ant *Crematogaster scutellaris* (Olivier) (Hymenoptera Formicidae) is a mixture of compounds, produced by the venom and the Dufour's gastral glands, known to act as a chemical contact poison applied to the enemy's body. Here we report a preliminary study where the sting secretion and the single dissected gastral glands from worker ants were tested for their antimicrobial activity. By the agar diffusion method, we demonstrated that sting secretion and both venom and Doufur's glands have a strong growth inhibition activity against Gram-positive and Gram-negative bacteria and entomopathogenic fungi. Moreover, we found that the inhibitory activity of sting secretion on *Bacillus subtilis* lasts in time for at least 24 hours from emission, with an increase after the first two hours.

Key words: *Crematogaster*, sting secretion, antimicrobial activity, gastral glands.

Introduction

Production of antimicrobial substances is not only an important aspect of innate individual immunity of organisms (Otti *et al*., 2014) but in social insects it represents also a significant component of the so called "social immunity" (Cremer *et al*., 2007; Schlüns and Crozier, 2009). This is especially true when chemicals are used for preventing pathogens or opportunistic microorganisms to spread in the nest environment (Baracchi *et al*., 2011; Tragust *et al*., 2013; Tranter *et al*., 2014) and to attack defenceless brood. Social insects are chemical factories (Billen and Morgan, 1998) and various exocrine glands have been described to produce antimicrobial secretions. In ants this function is particularly attributed to the metapleural glands (Yek and Muller, 2011) but other glands have been indicated as the source of antimicrobic or antifungine substances. For example mandibular glands produce such substances in *Calomyrmex* sp. (Brough, 1983) and in some species of *Atta* leaf cutter ants (Marsaro *et al*., 2001; Rodriguez *et al*., 2008; Mendonça *et al*., 2009), while a very active compound named iridomyrmecin is produced by the anal glands of the abdomen of the ant *Linepithema humile* (Mayr) (Pavan and Nascimbene, 1948). The venom of Hymenoptera often contains antimicrobial agents (Baracchi and Tragust, 2015; Moreau, 2013) and in social species this secretion can be used to defend the colony not only from predators or competitors but also from more subtle enemies such as pathogens. In honey bees and *Polistes* wasps (Turillazzi *et al*., 2006; Baracchi *et al*., 2011) venom is usually spread on the nest surface or in overwintering sites and the same happens in various ants such as fire ants (Story *et al*., 1991; Vander-Meer and Morel, 1995) while *Lasius neglectus* Van Loon, Boomsma et Andrasfalvy use their venom to protect their brood from diseases (Tragust *et al*., 2013). The antimicrobial activity of venom from bees, wasps and ants was recently reviewed by Fratini *et al*. (2017).

The genus *Crematogaster* is one of the largest of the family Formicidae with 427 valid described species according to the last report of Integrated Taxonomic Information System (ITIS, 2001). These are for the most part arboreal and common in tropical areas and many of them have a dominant role in the ant communities in a wide variety of microhabitats. *Crematogaster* present a sting with a particular spatula or spoon tip which is used to apply a drop of secretion to the body of enemies, usually represented by other ants. In *Crematogaster scutellaris* (Olivier) the secretion is actually a mixture of compounds produced by the venom glands and by a particularly enlarged Dufour's gland (Pasteels *et al*., 1989). The secretion is used as an insecticide and a repellent towards other ants (Marlier *et al*., 2004) and is emitted as a chemical weapon in any particular situation when the workers feel themselves or the colony in danger. In a more recent paper, however, also antimicrobial activity against Gram-positive and Gram-negative bacterial strains of the sting secretion has been demonstrated in one species (*Crematogaster pygmaea* Forel) from Brazil, supporting the claim that ant venom can be multifunctional (Quinet *et al*., 2012). The aim of this study was to assess if an antimicrobial function could be present also in the sting secretion produced by the very common Mediterranean acrobat ant *C. scutellaris*. In our study we performed preliminary growth inhibition tests on four bacteria (two Gram-positives, *Bacillus subtilis* and *Staphylococcus aureus* and two Gram-negatives, *Escherichia coli* and *Pseudomonas aeruginosa*) and on two entomopathogenic fungi (*Metarhyzium anisopliae* and *Beauveria bassiana*) using sting secretion and dissected Dufour's and venom glands. Moreover, we tested the timing of the inhibitory activity of sting secretion on *B. subtilis* up to 24 hours from its collection.

Materials and methods

Ant collection and treatment

Workers of *C. scutellaris* were collected from field colonies in the surroundings of Florence, Central Italy, in the spring of 2015 and 2016. 170 workers were kept in plastic boxes at the room temperature of 25-18 °C (L-D period 12-12 hrs) and relative humidity around 75% and fed with sugar and water until the milking of their sting secretion. 30 workers were kept in freezer at −20 °C and then killed with acetic ether just before their dissection to obtain Dufour's and venom glands.

Milking of the drops of sting secretion

Single alive workers were kept with sterile forceps at the level of petiole until a white drop appeared at the tip of their stings, that occurs almost immediately after seizing. The drop was collected with a Pasteur pipette glass capillary (reduced to 0.2 mm in section) and then blown out directly on the culture plate for the antibiotic sensitivity tests.

Dissection of Dufour's and venom glands

We deposited freshly killed ants in a drop of sterilized distilled water on a microscope glass and gently pulled the tip of their gaster with sterilized forceps to extract the main abdominal organs. We then collected the Dufour's gland and the venom glands + venom reservoir in order to use them directly for the antibiotic sensitivity tests.

Microorganisms and growth conditions

Bacillus subtilis 168 (Anagnostopoulos and Spizizen, 1961) and *Staphylococcus aureus* ATCC25923 were used as representatives of Gram-positive bacteria and *Escherichia coli* XL1Blue (Stratagene, La Jolla, CA, USA) and *Pseudomonas aeruginosa* ATCC27853 as representatives of Gram-negative bacteria, for antimicrobial tests. Cells of bacteria were grown aerobically at 37 °C, *E. coli, S. aureus* and *P. aeruginosa* in liquid or solid Luria Broth (LB) complex medium (Miller, 1972); *B. subtilis* 168 in Nutrient Broth and Nutrient Agar (OXOID™). The entomopathogenic fungi *Beauveria bassiana* ATCC 74040 (NATURALIS®) and *Metarhizium anisopliae* strain F52 (Met52®) were used as fungal indicators. They were grown in Malt Extract Broth and Malt Extract Agar (OXOID™) added with chloramphenicol 100 µg/ml as antibacterial.

Antimicrobial activity tests

We assayed microbial growth inhibition with the agar diffusion method as previously described (Turillazzi *et al*., 2004). Petri dishes of 9 cm diameter with solid medium were plated with a 0.1 ml suspension containing 10^5 bacterial cells from an overnight culture in liquid medium or 10^5 fungal spores. We performed antimicrobial tests by placing onto the plates 1) just collected drops of sting secretion that we blew out from the Pasteur pipette on the agar plate; 2) drops collected in the same way but tested at different times in order to check for their antimicrobial activity after collection; 3) freshly dissected glands (both venom and Dufour's ones). Ampicillin (5 µl drop of 100 µg/ml solution) and Nystatin (3 µl drop of 4000 µg/ml solution) were used as standards for bacteria and fungi, respectively. Plates were incubated at 37 °C for 24 h for bacteria and at 30 °C for at least 3 days for fungi.

Evaluation of antimicrobial activity

Antimicrobial activity against both bacteria and fungi was indicated by the clear halo of growth inhibition around the ant derivatives on the plates. In most cases we took note of the simple presence or absence of growth inhibition caused by experimental items on the target microorganism. To have an indication for changes of antimicrobial activity in time we calculated the area (in pixels) of the inhibition zone on photos of the Petri dishes taken with a digital camera using the free software ImageJ.

Results

Antimicrobial activity of the pure sting secretion

Drops of sting secretion just collected from the tip of the abdomen of 21 different workers of *C. scutellaris* gave inhibition of growth of *B. subtilis* in 17 cases. We had also a positive antimicrobial activity of 20 out of 20 sting secretion drops against *S. aureus* and of 20 out of 20 sting secretion drops against *P. aeruginosa* (figure 1). We had only 2 slight growth inhibition zones around 21 sting secretion drops deposited in the plates inoculated with *E. coli.* Ampicillin gave inhibition of all the tested bacteria.

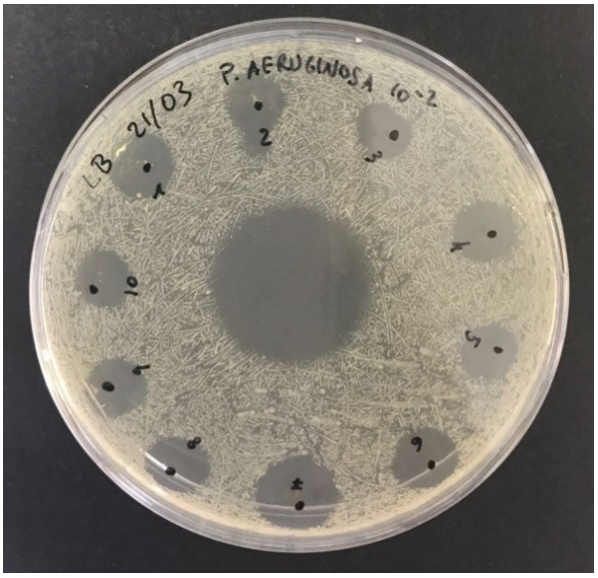

Figure 1. Growth inhibition activity of sting secretion of *C. scutellaris* workers on bacteria by agar diffusion method. Halos of growth inhibition of *P. aeruginosa* produced by drops of sting secretion collected from 10 different workers of *C. scutellaris.* The central inhibition halo was due to ampicillin used as control.

Table 1. Growth inhibition activity of sting secretion of *C. scutellaris* workers on bacteria and fungi.

Microorganism	Number of samples	
	Positive (inhibition)	Negative
B. subtilis	17	4
S. aureus	20	0
P. aeruginosa	20	0
E. coli	2	19
B. bassiana	8	2
M. anisopliae	2	8

Table 2. Progression in time of the growth inhibition activity of sting secretion on *B. subtilis* 168.

Time	Positive samples (inhibition)	Mean area and SD of growth inhibition halos	Negative samples
0	9	2.04 ± 1.08	1
1 hr	7	2.51 ± 2.04	3
2 hrs	18	5.05 ± 3.75	2
5 hrs	8	2.65 ± 1.76	2
24 hrs	9	3.02 ± 1.81	1

Drops of sting secretion freshly collected from ten workers and plated on plates inoculated with *B. bassiana* gave 8 out of 10 inhibition rings. We had only 2 out of 10 inhibition halos when we plated drops of sting secretion of ten different workers on plates inoculated with *M. anisopliae.* Nystatin gave always inhibition. Table 1 resumes the results for the various microrganisms.

To further check if the inhibitory activity of the secretion could decrease in time after emission we performed a test using *B. subtilis* 168. We collected drops from 60 workers from two colonies and kept them at room temperature. We plated 10 drops immediately, 10 after 1 hr, 20 after 2 hrs, 10 after 5 hrs and 10 after 24 hrs on plates inoculated with *B. subtilis 168.* The results after 24 hours incubation are reported in table 2 where the mean area of the inhibition halos is indicated in pixels.

We observe a substantial constancy in the antimicrobial activity in time but the action seems to increase after the very first two hours from the emission; the Kruskal-Wallis analysis of variance was significant (H = 10.61, P = 0.03) but post hoc tests, Bonferroni corrected, did not show any significant difference between the inhibition areas.

Antimicrobial activity of freshly dissected glands

Dufour's gland of *C. scutellaris* is quite developed and can be easily extracted at dissection. Venom glands and venom reservoir are quite smaller and sometimes we failed to dissect them. For this reason we could test the activity of an higher number of Dufour's glands (N = 24) of workers compared to that of the venom glands (N = 11).

We tested the glands on *B. subtilis* and we had growth inhibition in 22 out of 24 cases for the Dufour's glands and in 9 out of 11 cases for venom glands. Obviously the activity depended also on the quantity of secretion present in each gland that we did not measure.

Discussion and conclusions

The main function of the sting secretion of *C. scutellaris* has been considered as a chemical weapon of these ants to be especially used, as contact poison, in competitions against other ants. Its composition has been studied by Douloze *at al*. (1986; 1991) and by Pasteels *et al*. (1989) who described the chemical process by which the compounds of the quite enlarged Dufour's gland mix on the spatulate sting and are activated by enzymes produced by the venom glands, forming highly electrophylic aldehydes which are more toxic than the pure secretion of the Dufour gland itself. Together with the high toxicity also an interspecific repellency, which make ants of other species strongly and immediately repelled after a contact between their antennae or mouthparts with the sting secretion, was described by Marlier *et al*. (2004). Our findings demonstrated for the first time that sting secretion of this species has also antibacterial and antimycotic activities and that also the pure secretions of the glands which contribute to its formation present such properties. Moreover, to our knowledge this is the first time that antimicrobial activity is reported for the secretion of the Dufour's gland alone in Hymenoptera Aculeata.

The toxic properties of the sting secretion and its use as a contact poison were also reported for three other species of neotropical *Crematogaster* (*C.* sp. prox. *abstinens*, *C. distans* and *C. brevispinosa rochai*) by Heredia *et al*. (2005) and for three species from New Guinea (Leclercq *et al*., 1997) but antibacterial activity was previously demonstrated only for the sting secretion of the neotropical species *C. pygmaea*, while that of other two tested species (*C. distans* and *C. rochai*) did not show any antimicrobial property (Quinet *et al*., 2012).

Actually other researches previously reported the presence of antimicrobial substances in species of the genus *Crematogaster* but these studies used extracts of all the ant bodies to test their action against microorganisms. Pavan and Nascimbene (1948), for example, reported a marked antimicrobial activity of *C. scutellaris* body extracts in ethyl ether against Gram-negative and Gram-positive bacteria and Matiz Melo and del Rosario Osorio Fortich (2013) did the same for an undetermined species from Colombia which they tested in ethanolic extracts. In these cases we cannot be sure that venom could have been the source of antimicrobial agents as other glands, especially the metapleural ones (the secretion of which in *C. scutellaris*, as in many other ants, presents antimicrobial activity (Maschwitz *et al*., 1970; Yek and Muller, 2010), could have contributed to the action of the entire body extracts.

Sting secretion of *C. scutellaris* shows inhibition activity against all the target microorganism we tested, espe-

cially against Gram-positive bacteria and the entomopathogenic fungus *B. bassiana*. The negative samples in our tests could be due to differences in amount and/or timing of antimicrobials production by individual ants and their glands. On the other hand, since we do not know the chemical nature of the antimicrobial substance(s), we cannot exclude a different susceptibility of the tested microorganisms (see results for *E. coli* and *P. aeruginosa*, both Gram-negative bacteria, in table 1). Both pure Dufour's gland secretions and venom are active against *B. subtilis*. Dufour's gland is particularly enlarged in this species and produces the most part of the sting secretion. The action of sting secretion lasts in time for at least 24 hrs and seems to have more activity after two hours from emission which could be just due to the formation of the active compounds in the chemical reaction which occurs on the sting (Pasteels *et al*., 1989). It is possible that the venom and Dufour's gland secretions are used both independently or together according to different necessities. Workers usually stay in the nest keeping the sting slightly extruded from the tip of the abdomen and it is often possible to observe very tiny drops of secretion on it (Turillazzi, unpublished). It may be that this is a way to produce volatile compounds that serve to sanitize the nest environment similarly to what happens in colonies of *Solenopsis invicta* Buren (Obin and Vander Meer, 1985; Wang *et al*., 2015). Quinet *et al*. (2012) put in relation the presence of antimicrobial activity in the sting secretion of *C. pygmaea* and its absence in *C. distans* and *C. rochai* with the fact that only the first species is an arboreal one: this could be true also for *C. scutellaris* but a general ecological explanation needs to be supported by data on other species.

LC and MALDI TOF mass spectrometry analyses are in progress to determine the characteristics of the medium and high mass weight components of the secretions.

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