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### Alkaloid secretion inhibited by antibiotics in *Aphaenogaster* ants

*La sécrétion d'alcaloïdes inhibée par un traitement antibiotique chez les fourmis Aphaenogaster*

Alain Lenoir\*, Séverine Devers

IRBI, Institut de recherche sur la biologie de l'insecte, Université de Tours, Faculté des sciences, parc de Grandmont, 37200 Tours, France

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#### ABSTRACT

Although alkaloids are frequent in the poison glands of ants of the genus *Aphaenogaster*, this is not the case for *A. ibérica*. Hypothesizing that in the genus *Aphaenogaster*, alkaloids are produced by symbiotic bacteria, except for *A. ibérica*, we treated an experimental lot of both *A. ibérica* and a 'classical' *Aphaenogaster* species, *A. senilis*, with an antibiotic. Compared to workers from a control lot, this treatment reduced considerably alkaloid production in *A. senilis*, whereas *A. ibérica* did not react to the treatment. Furthermore, the treatment induced an increase in cuticular hydrocarbon quantities in *A. senilis*, but not in *A. ibérica*. An analysis of the ant microbiota will be the next step to confirm our hypothesis. © 2018 Published by Elsevier Masson SAS on behalf of Académie des sciences. This is an open access article under the CC BY-NC-ND license (<http://creativecommons.org/licenses/by-nc-nd/4.0/>).

#### RÉSUMÉ

On trouve de nombreux alcaloïdes dans les glandes des fourmis, particulièrement chez les *Aphaenogaster*, mais pas chez *A. ibérica*, qui fait exception. Nous avons émis l'hypothèse que la production d'alcaloïdes pouvait être liée à l'activité de bactéries qui seraient absentes chez *A. ibérica*. Pour cela, nous avons traité les fourmis avec un antibiotique. Chez *A. senilis*, qui représente le cas « classique », la production d'alcaloïdes chute considérablement, alors que le traitement est sans effets sur *A. ibérica*. Le traitement induit aussi une augmentation des hydrocarbures cuticulaires chez *A. senilis*, mais pas chez *A. ibérica*. Ces données indiquent un rôle bactérien, le microbiome de ces fourmis restant à étudier.

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\* Corresponding author.

E-mail addresses: [alain.lenoir@univ-tours.fr](mailto:alain.lenoir@univ-tours.fr) (A. Lenoir), [severine.devers@univ-tours.fr](mailto:severine.devers@univ-tours.fr) (S. Devers).

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## 1. Introduction

The poison gland of many myrmicine ants, including those of the genus *Aphaenogaster*, contain alkaloids [1,2]. This is the case for *Aphaenogaster senilis* (i.e. poison gland containing anabasine, anabaseine, and pyridines), but not for *A. ibérica* [3]. As these two species are phylogenetically closely related, this difference is very surprising, but it is probably linked to the differences in their habitat, which explains why *A. senilis* uses alkaloids in trail pheromone [3].

Because the importance of microbiota was highlighted in the biology of organisms, including insects and among them ants [4,5], we hypothesized that alkaloid production in *A. senilis* may be due to a bacterial activity, something supposedly absent in *A. ibérica*. We thus treated the workers of the two species with Rifampicin, an antibiotic frequently used in insects, including ants [6,7]. To test this hypothesis, we measured the quantities of substances in poison and Dufour glands of the two above-cited *Aphaenogaster* species as well as the effect of the treatment on cuticular hydrocarbons to investigate a more general effect on the ants.

## 2. Material and methods

We collected in Spain four colonies of *A. senilis* in Doñana National Park (Las Beles, 36°58'53"N, 6°29'11"W), and two colonies of *A. ibérica* in the Sierra Nevada (37°08'67"N, 3°28'33"W, 1370 m). All colonies had a queen and abundant brood. They were maintained in artificial nests composed of a plastic box containing two to three tubes half-filled with water. Each nest was connected through a plastic tube to a large foraging arena, where food (mealworms and honey) was provided *ad libitum*. The room temperature was maintained at 25 °C.

For the experiments, we prepared small plastic boxes (i.e. foraging arena) with a tube provided with a watering place (i.e. the nest) and placed inside 100 workers with some brood. The ants were fed three times a week during one month with honey, whereas mealworms or maggots and pieces of orange were offered *ad libitum*. We made control groups (C) and groups treated with the antibiotic Rifampicin

(R) as a 50 µg/ml solution were mixed with honey (4 × 2 groups for *A. senilis* and 2 × 2 groups for *A. ibérica*).

The ants were treated for one month, which is efficient to kill bacteria in *Camponotus* [7]. The workers were frozen at –20°. Cuticular hydrocarbons were extracted from the thoraxes of workers placed in vials with 0.5 ml of pentane during 1 h, and then the thoraxes were removed whereas the solvent was evaporated. The residue was re-dissolved in 50 µL of pentane, of which 2 µL were injected into a PerkinElmer GC/MS device using a DB-5 fused silica capillary column; temperature programmed from 150 °C (5 min hold) to 300 °C at 10 °C/min, and held at 300 °C for the last 10 min.

We also dissected poison glands and Dufour glands of each species and maintained them during 1 hour in 50 µL pentane, of which 2 µL were injected into the PerkinElmer GC/MS device (for the number of glands dissected, see Table 1). Separations were achieved using the same DB-5 fused silica capillary column; temperature programmed from 50 °C (2 min hold) to 300 °C at 6 °C/min, and held at 300 °C for the last 10 min [3]. In all extracts, a C20 was used as an internal standard to quantify the quantities of alkaloids and hydrocarbons.

We used Mann–Whitney's *U* test to compare the sample quantities and a cluster analysis (Ward method, Euclidean distances) to compare the hydrocarbon profiles (Statistica8 Software).

## 3. Results

### 3.1. Cuticule

Because there was very little worker size variation between both the colonies and species, we measured the hydrocarbon quantities per worker rather than per mg of fresh ant (Fig. 1).

Concerning *A. senilis*, the quantity of hydrocarbons per thorax of control workers was significantly lower than that of workers treated with the antibiotic ( $n = 10$  in both cases; median, lower and upper quartiles; 237 ng; 231.6 ng, 376.9 ng versus 610.7 ng; 356.9 ng; 1159.9 ng;  $U = 9$ ;  $p = 0.001$ ). Comparatively, we did not record significant differences for *A. ibérica* workers ( $n = 5$  in both cases;

**Table 1**

Poison glands quantities (ng/gland) for total content and alkaloids, Dufour glands total substances in the two species *A. senilis* and *A. ibérica* for controls and treated with Rifampicin.

	Control				Rifampicin				<i>p</i>
	Median	Lower quartile	Upper quartile	<i>n</i>	Median	LQ	UQ	<i>n</i>	
<i>Aphaenogaster senilis</i>									
Poison gland total substances	383.8	77.9	1264.0	18	90.4	55.0	391.0	20	0.11
PG alkaloids only	289.0	34.6	1089.8		17.3	4.6	217.9		0.008
Dufour gland total substances	842.5	394.0	1441.1	17	1827.7	102.5	3224.8	15	0.500
DG alkaloids only	0				0				
<i>Aphaenogaster ibérica</i>									
Poison gland total substances	41.6	6.8	77.3	10	78.9	45.7	127.0	12	0.090
Dufour gland total substances	23.2	5.0	33.9		38.5	24.0	65.1		0.140
<i>U</i> -test <i>A. senilis</i> / <i>A. ibérica</i>									
Poison gland total substances	0.001								
Dufour gland total substances	< 0.001								

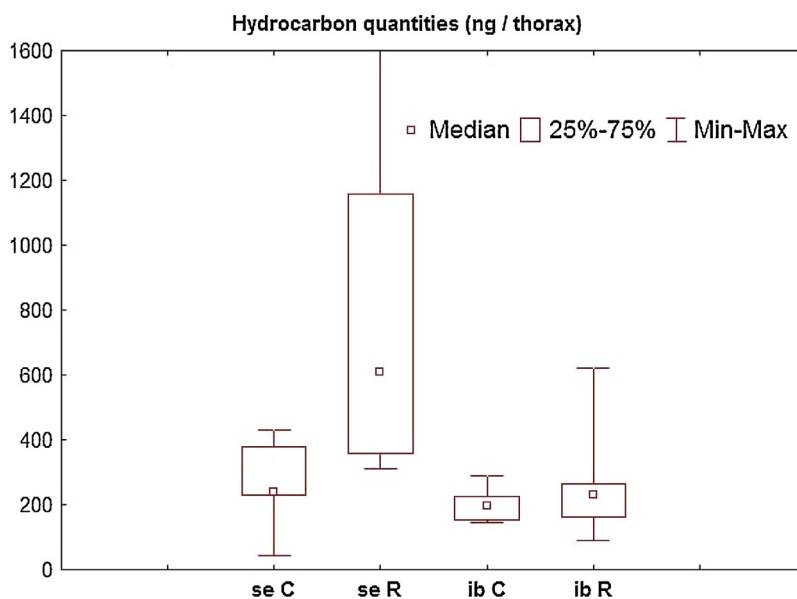


Fig. 1. Hydrocarbon quantities (ng/thorax) in *Aphaenogaster* ants. se: *Aphaenogaster senilis*, ib: *Aphaenogaster iberica*, C: control, R: Rifampicin treatment. se R is significantly different from the other groups.

196 ng; 152 ng; 225 ng versus 230 ng; 160 ng; 263 ng for the controls and the treated ants, respectively;  $U = 10$ ;  $p = 0.69$ ). Furthermore, no difference appeared between the controls of the two species ( $U = 13$ ;  $p = 0.16$ ).

A cluster analysis of the hydrocarbon profiles did not indicate a difference between control and treated ants in both species (figures not presented). It also showed that the antibiotic treatment increases the production of hydrocarbons in *A. senilis* without changing the profile, and had no effect on *A. iberica*.

### 3.2. Glands

The *A. senilis* poison glands contain alkanes (mean: 3%), alkenes (6.6%), aldehydes (2.6%), and a great quantity of alkaloids (87.5%) as previously presented in [3], whereas the Dufour glands, devoid of alkaloid (0%), contain alkanes (51%), alkenes (47%), and 1% of aldehydes (Table 1).

The *A. iberica* poison glands contain alkaloids (6.1%), alkanes (24.5%), alkenes (62.1%), and small quantities of aldehydes (3%), the composition of the Dufour gland being quite similar, containing alkaloids (1.8%), alkanes (38.4%), alkenes (54.6%), and aldehydes (3.1%).

The quantities of substances in the glands are given in Table 1. The poison glands of *A. senilis* are significantly bigger than those of *A. iberica* and contain more substances ( $n = 18$  and  $n = 10$  respectively; median, lower and upper quartiles; 383.8 ng versus 41.6 ng; for *A. senilis* and *A. iberica*, respectively;  $U = 25$ ;  $p = 0.001$ ). The same is true for the Dufour glands ( $n = 17$  and  $n = 10$ , respectively; 842.5 ng versus 23.2 ng for *A. senilis* and *A. iberica*, respectively;  $U = 0$ ;  $p < 0.001$ ).

The total content of the *A. senilis* poison gland did not change after the antibiotic treatment ( $n = 18$  and  $n = 20$ , respectively; 383.8 ng versus 90.4 ng,  $U = 125$ ;  $p = 0.11$ ; Table 1). If we consider the classes of substances, there is

no difference for alkanes ( $U = 163$ ;  $p = 0.63$ ), alkenes ( $U = 168$ ;  $p = 0.74$ ), and aldehydes ( $U = 177$ ;  $p = 0.94$ ), but the alkaloid quantities were significantly higher in the control compared to workers treated with the antibiotic (289 ng versus 17.3 ng for controls and treated ants, respectively;  $U = 90$ ;  $p = 0.008$ ; Fig. 2), representing a decrease of 94%.

Still concerning *A. senilis*, the total content of the Dufour gland increased after the Rifampicin treatment (842.5 ng versus 1827.7 ng for the control and the treated ants, respectively;  $U = 109$ ;  $p = 0.50$ ; Table 1).

The content of the *A. iberica* glands did not change with the treatment (Table 1; poison gland: 41.6 ng versus 78.9 ng;  $U = 34$ ;  $p = 0.09$ ; Dufour glands: 23.2 ng versus 24.0 ng;  $U = 37$ ;  $p = 0.14$ ).

## 4. Discussion

We observed a general effect of the antibiotic in *A. senilis* with an increase in the cuticular hydrocarbon quantities likely corresponding to a reaction of the ants to the stress induced by the decrease in bacteria quantities. This is reminiscent of the case of *Camponotus* ants that have mutualistic endosymbiotic bacteria with alimentary and immune functions [7]. Here too, the antibiotic treatment results in higher production of cuticular hydrocarbons compared to controls, plus a melanization of the cuticle [8]. In *Camponotus* spp. as well as *A. senilis*, the cuticular hydrocarbon profiles are not modified by the antibiotic treatment, but by a larger quantity of hydrocarbons produced, likely in response to a decrease in the number of symbiotic bacteria.

We also observed a spectacular effect of the antibiotic on the production of alkaloids by the poison gland of *A. senilis* with a decrease of 94%. This can be due to a direct production by bacteria which may be endosymbiotic in the

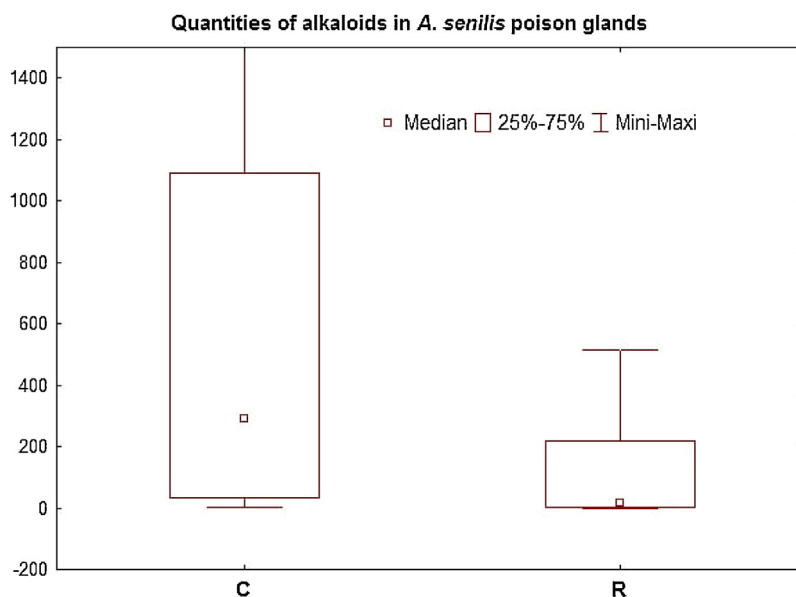


Fig. 2. Alkaloid quantities (ng/gland) in poison glands of *Aphaenogaster senilis*. C: control, R: Rifampicin treatment. The difference is highly significant ( $p=0.008$ ).

membrane of the poison gland or by other bacteria in the ant body acting on the synthesis pathway of the alkaloids. In their review on toxins, Touchard et al. [9] said that “It is also possible that a microbial symbiont may have been involved in the production of intermediary compounds, as was suggested for some alkaloids found in sponges and briefly hinted at in ants by Saporito et al. [10].” Also, the production of pyrazines depending on bacteria was noted in Attine ants [11], and it was suggested by different researches in ant-fungus species [12,13]. Nevertheless, the production of alkaloids by free bacteria was noted only in a few marine species [14].

*Aphaenogaster iberica* differs from *A. senilis* in its geographical distribution and its mode of recruitment of nestmates [15]. We saw here that these two ant species also differ in their cuticular hydrocarbons and glandular contents, *A. iberica* lacking alkaloids, or they are present only as traces [3]. The fact that *A. iberica* did not react to the antibiotic treatment is likely due to the absence of symbiotic bacteria in this species. More research is necessary to know in detail the microbiome of these ant species.

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