Dufour’s gland secretion as a repellent used during usurpation by the slave-maker ant *Rossomyrmex minuchae*

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Abstract

In slave-making ants, the invasion of the host colony by newly mated queens is a critical stage. We studied the strategy used by *Rossomyrmex minuchae* queens to invade their host *Proformica longiseta*. Field observations revealed that queens enter the host nest unchallenged by the host workers in the vicinity of the nest entrance. Pre-usurpation queens were found to possess a highly inflated Dufour’s gland, which considerably reduces in size after successful usurpation. Chemical analysis of these queen glands revealed tetradecanal to be the major product in pre-usurpation *Rossomyrmex* queens, but to be almost absent in queens that have been adopted by *P. longiseta*. We consequently hypothesized that tetradecanal is a repellent that is used by queens to prevent host worker aggression. We tested its repellent effect by attempting to deter starved, highly motivated workers from a droplet of honey. Tetradecanal indeed proved to be highly repellent both to host worker *P. longiseta* and non-host worker *Formica selysi*. It was even more powerful than limonene, a reported general ant repellent. These results are consistent with the hypothesis that *R. minuchae* queens use Dufour’s gland secretion as a weapon during nest usurpation. The general use of tetradecanal as a defensive compound, and its seemingly non-specific repellent effect on ants, indicate that it may act as a general ant repellent. Its adoption by *R. minuchae* queens thus provides them with an efficient defensive and offensive chemical weapon during their long and risky search for new host nests.

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1. Introduction

Obligatory social parasites must overcome two host barriers: during usurpation of the host colony by the queen, and during social integration of the queen thereafter. Upon establishment in the new nest, the slave-maker workers become further engaged in raids, the success of which depends again on crossing the barrier of host resistance. Many parasites use behaviour-modifying chemicals to evade host resistance (reviewed in Lenoir et al., 2001). Two general strategies are recognized: deceiving the host through chemical mimicry and repelling the host by emitting aversive substances. Queens of the ant *Bothriomyrmex syrius*, for example, produce large quantities of 6-methyl-5-hepten-2-one in their pygidial gland, a substance that is also the major alarm pheromone component of its host *Tapinoma simrothi*. This compound is not present in *B. syrius* workers, indicating that the queen alone uses it while usurping the host nest (Lloyd et al., 1986). The chemical composition of Dufour’s gland secretion of the European slave-maker ant *F. sanguinea* bears high similarities with that of its hosts, *F. fusca* and *F. rufibarbis*, and was suggested to assist the slave-maker during raids (Bergström and Löfqvist, 1968). This was...
experimentally shown a few years later in *F. sanguinea* population in North America, where it parasitizes *F. subsericea* and was termed the “propaganda pheromone” (Regnier and Wilson, 1971). Similar use of a “propaganda pheromone” was also shown in behavioural studies of the slave-maker ant *Harpagoxenus sublaevis* in its raids on its host species *Leptothorax acervorum* (Allies et al., 1986). A later study of Dufour’s gland chemistry in both the slave-maker and the host (Ollett et al. (1987) for *H. sublaevis* and Ali et al. (1987) for *L. acervorum* revealed that both possess heptadecadiene and heptadecene as major constituents, suggesting that these are the basis for the successful mimicry.

The second strategy used by slave-maker ants is the use of repellents to prevent or nullify host aggression during nest usurpation by the slave-maker queen, or during raids by her workers. The thief ant *Solenopsis (Diploroptrum) fugax* uses 5-buty-2-heptylpyrrolidine, released from the poison gland during raids against other ant species (Blum et al., 1980). Dufour’s gland secretion is used by queens of the slave-maker species *Polyergus breviceps* and *P. rufescens* as a source of offensive chemicals that decrease the aggressive response of the host *Formica* workers during usurpation (Topoff and Zimmerli, 1993; D’Ettorre et al., 2000; Mori et al., 2000; Visicchio et al., 2000). Accordingly, the gland reveals hypertrophy in pre-usurpation queens, but decreases in volume shortly after she has successfully invaded the host colony and been adopted by its resident workers. The social parasite ant *Leptothorax kutteri* also uses a hypertrophied Dufour’s gland as a source of effective repellent during nest usurpation (Allies et al., 1986), but the chemical structures of the relevant compounds are still unknown. The bumblebee social parasite *Bombus (Psithyrus) norvegicus* seems to use the same strategy when invading nests of *B. hypnorum*. In its enlarged Dufour’s glands it produces dodecyl acetate that has a strong repellent effect on workers of the host (Zimma et al., 2003).

In the present study we explored the role of Dufour’s gland secretion in host-nest usurpation by queens of the slave-maker ant *Rossomyrmex minuchae*. This is a rare, endemic species that occurs in the high mountains of the Sierra Nevada in Spain, where it specifically parasitizes nests of *Proformica longiseta* (Ruano, 2000; Ruano and Tinault, 1999, 2005). Mated queens disperse solitarily in search of a new host nest, a process that may take several hours. During this period, the queen is theoretically exposed to high predation risks as well as aggressive defence by host workers when she attempts to usurp their nest. Nevertheless, field observations during the process of usurpation revealed that the usurping queen is able to penetrate the host’s nest unchallenged, despite the presence of host workers near the nest entrance. Dissection of queens before usurpation revealed an extraordinarily inflated Dufour’s gland. We analysed the glandular secretion and tested the hypothesis that it contains a repellent that is used to deter and pacify workers during nest usurpation.

## 2. Material and methods

### 2.1. Ants

*P. longiseta* and *R. minuchae* were collected in Sierra Nevada National Park (Granada, Spain). Queens of *R. minuchae* before usurpation (n = 6) were collected as they exited their natal nest, and those after usurpation (n = 2) were excavated from parasitized *P. longiseta*, between 1998 and 2001 (*R. minuchae* is a scarce and protected species, included in the IUCN Red List of Animals in Danger of Extinction). Fragments of 12 non-parasitized colonies of *P. longiseta* were collected from various sites at the Sierra Nevada Park in June 2003 and 2004 and kept in the laboratory until the experiments. *Formica selysi* were obtained from laboratory colonies, originally collected in Morillon, Haute-Savoie, French Alps.

### 2.2. Chemical analyses and synthesis

For chemical analyses, Dufour’s glands from various castes and species were cleanly dissected under water and extracted with pentane. An aliquot was analysed by GC/MS (GC 8000 linked to a quadrupole mass spectrometer MD 800—Fisons Instruments, Mainz, Germany) using a DB1 fused silica capillary column (25 m; 0.25 mm id, 0.25 μm film thickness, J & W-Scientific) that was temperature programmed from 60 °C (5 min hold) to 320 °C at 5 °C/min. Helium served as the carrier gas. Structure elucidation was achieved by comparison with published mass spectra (McLafferty and Stauffer, 1989; Doolittle et al., 1995). Positions and configurations of double bonds in unsaturated compounds were not determined. Quantification of the glandular secretion was performed under the above conditions after adding 80 μg of 2-decenal as an internal standard to each sample.

Commercially available tetradecanal is largely represented by its trimer, which cannot easily be hydrolysed to yield the pure monomer. Therefore, tetradecanal was synthesized starting from 1-pentadecene (Aldrich) by ozonolysis in methanol (Razumovskii and Zaikov, 1984). The resulting tetradecanal was purified by column chromatography on silica (Merck 60, 230–400 mesh) using hexane/ethylacetate. The overall yield was 90%, and the purity of the product was 99.5%, as proven by GC/MS (McLafferty and Stauffer, 1989) as well as ¹H NMR spectra and ¹³C NMR spectra.
2.3. Repellence tests

The repellence tests were conducted according to a previously published protocol (D’Ettorre et al., 2000). Briefly, starved ants were offered a droplet of honey on a glass coverslip (2 cm × 2 cm; feeding slide) in a neutral arena (Petri dish, 9 cm diameter). The bottom of the dish was covered with filter paper, which was changed after each test. The coverslip was evenly applied with 10 μl of the treatment solution or pentane as solvent control, onto which the honey droplet was deposited. Treatment solutions included various concentrations of either tetrade canal (the major constituent of Dufour’s gland secretion of Rossomyrmex queens) or a racemic mixture of limonene, a known ant repellent (Honda, 1983; Scheffrahn et al., 1987) that was used for validating our repellence test.

Before each test, the ants were placed in a glass tube within the arena for 1 min to acclimate, and removing the glass tube started the test. We recorded the time elapsed until the ant reached and imbibed the droplet (latency to feeding), which served as the repellence criterion. If the ant did not feed on the honey within 2 min, it was re-exposed to a new, non-treated honey droplet to verify its starved status. Ants that did not feed the second time were discarded. Most of the experiments were performed blind: the person observing the behaviour was not aware of the type of treatment. A total of 333 tests were performed with P. longiseta originating from 12 different colonies, of which only 20% of the ants were discarded as non-starved. For F. selysi, 198 tests were performed using ants from four colonies, none of which were discarded as non-starved.

Colonies of P. longiseta contain many replete-workers that store sugar in their abdomen, which they regurgitate in times of need (Bernard, 1975). The colonies were therefore starved for as long as 1 week before the test to assure food shortage in the colony. To further assure starvation we isolated groups of ants (medium size non-replete) from these colonies 2 days before the test in a Petri dish with moistened filter paper only. The colonies of F. selysi were starved for 3 days only.

Statistical analyses were made using Kruskal–Wallis test on the medians because the ants that did not reach the food were given an arbitrary score of 120 s, the duration of the observation. Multiple post-hoc compar-

ison tests between groups were also made with Kruskal–Wallis (Statistica® software).

3. Results

3.1. Chemistry

Dufour’s gland in R. minuchae queens before usurpa-
ton revealed hypertrophy; the glandular secretion was composed of a series of aliphatic aldehydes of which tetrade canal represented the major constituent (617 ± 244 μg tetrade canal/gland (mean ± SD, n = 6) over 80% of the secretion) followed by dodecanal and minor amounts of higher molecular weight aldehydes. These were accompanied by mostly unsaturated hydrocarbons of which heneicosane dominated (Fig. 1A). There were no apparent qualitative or quantitative differences between virgin and mated queens before usurpation, but the low sample size did not allow for statistical comparison. In contrast, the gland volumes in queens after usurpation were greatly reduced, and showed a considerably altered composition of volatiles. The aldehydes had almost disappeared, together with a dramatic decrease in the amount of tetrade canal (Fig. 1B). In these post-usurpation queens the secretion was dominated by hydrocarbons, including a series of alkenes (nonadecene to hentriacontene) of which heneicosane was the major compound. The contents of the Dufour’s gland in workers resembled that of queens after usurpation, being mostly comprised of hydrocarbons, with only minor amounts of tetrade canal. The workers differed from the queen in possessing alkanes (with tricosane dominating) rather than alkenes as the major hydrocarbons. Although the hydrocarbon composition of the glandular exudates bore some similarities to cuticular hydrocarbons (A. Hefetz, unpublished data), it was not identical. Furthermore, the glands were cleanly dissected without any traces of cuticle (except for the cuticular intima enveloping the glandular reservoir), supporting the glandular origin of these hydrocarbons. Analysis of Dufour’s gland secretions of queens and workers of P. longiseta revealed similar patterns of low-molecular weight hydrocarbons, among which tridecane, 3-methyltridecane and pentadecane constituted major compounds (Fig. 2A and B). No
traces of aldehydes, characteristic of *R. minuchae* queens, were found.

### 3.2. Repellence tests

*P. longiseta* workers in the control tests reached the honey droplet slide in 25.5 s (median) and started feeding (Table 1). As there were no differences in the reaction to tetradecanal between the various nests, the results were pooled. The feeding slides treated with high concentrations of tetradecanal (between 30 μg/μl (1/2 gland equivalent) and 500 ng/μl (1/120 gland equivalent)) were extremely repellent; most of the ants approached the honey droplet, but avoided contact. When reaching the treated slide the ants frequently retreated rapidly and cleaned their antennae. On several occasions when the ants walked on the tetradecanal treated glass at these high concentrations, they remained immobile, appearing anaesthetized for long periods (up to 10 min). At a concentration of 10 ng/μl (1/6000 gland equivalents; 0.25 ng/mm²) tetradecanal was still significantly more repellent than the control (45% of the ants did not reach the honey), but the reaction of the ants was not so violently repulsive. At a concentration of 1 ng/μl (0.025 ng/mm²) their reaction did not significantly differ from that towards the pentane control (P = 1), but 30% of the ants still avoided the substance barrier.

To verify whether the repellent effect of tetradecanal is specific to *P. longiseta*, we also tested workers of *F. selysi*. These workers were more mobile and reached the honey droplet within 8 s. However, they were also repelled by tetradecanal at a threshold level that was identical to that of *P. longiseta*: 1 ng/μl (Table 1, P = 1). Racemic limonene was highly repellent to both *P. longiseta* and *F. selysi*, with a threshold of 50 ng/μl (Table 1, P = 1).

### 4. Discussion

Host-nest usurpation and killing of the resident host queen is an obligatory step in nest foundation of the slave-making ant *R. minuchae*. Recently mated queens exit the nest solitarily in search of a new host nest (Ruano and Tinaut, 2005), and are therefore exposed both to predation and antagonism by the host workers. The means by which the queen protects herself is by employing a powerful repellent, produced by the Dufour’s gland. Queens before usurpation contain extraordinarily large amounts of tetradecanal in this gland (~600 μg), while glands of queens that have successfully usurped a nest, or those of workers, contain
only small quantities of this substance. We believe that
this small amount of tetradecanal in these latter queens
represents the residue of non-ejected secretion during
usurpation, or a considerably reduced biosynthesis. In
this study we tested the hypothesis that tetradecanal is a
potential repellent against host workers. In our bioassay
we attempted to repel highly starved, and therefore
motivated, ants from a drop of honey. Under these
conditions, tetradecanal was shown to be a powerful
repellent against host worker *P. longiseta*, as well as
against non-host worker *F. selysi*. Although the present
study was limited to only two species, the results suggest
that tetradecanal might constitute a general insect
repellent. The host workers are apparently very sensitive
to this substance, with its threshold repellent effect being
10 ng/μl (equivalent to 0.25 ng/mm² in our experiment).
This comprises 1:6000 of the amount found in the gland,
more than sufficient both for deterring potential
predators and defending host workers. This result is
consistent with field observations that searching females
experience low predation rates, despite long search
periods (Ruano, 2000), and that host workers ignore
usurping females, which enter the host nest through the
main nest-entrance unchallenged (Ruano, unpublished
observations). Interestingly, it was observed that at high
concentration tetradecanal had an anesthetic effect
on *P. longiseta* workers, which may explain why the
ants remain immobile even when the queen enters the
host nest. Tetradecanal was even more effective than
limonene, reported as a general insect repellent (Wood
et al., 2000), specifically as an ant repellent (Honda,
1983; Scheffrahn et al., 1987; D’Ettorre et al., 2000) or
even as an insecticide (Quilico et al., 1960). This not only
validates our bioassay, but also underlines the repellent
potency of tetradecanal.

Tetradecanal seems to be widely used as a defensive
secretion in insects. It was isolated from secretions of
hemipterans, beetles and cockroaches (Blum, 1981). In
ants it has been found in the Dufour’s gland of
*Pristomyrmex*, where its role is unknown (Billen et al.,
2000). Its wide use as a defensive secretion and its
powerful repellent effect on both a host and non-host
species lend credence to the hypothesis that tetradecanal
may be a general ant repellent, adopted by *R. minuchae*
queens as an offensive weapon during usurpation. The
copious amount produced, reflecting a large energetic
cost, seems to be essential since *R. minuchae*
queens, unlike for example *Polyergus*, do not usurp new
nest during raids, and therefore lack the protective cover of
the raiding workers. The fact that different slave-maker
ants use different chemical arsenals in order to deter
their hosts indicates a convergent evolution of this
character in the formicine parasites.

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