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SHORT COMMUNICATION

Response of workers of *Atta sexdens rubropilosa* (Hymenoptera: Formicidae) to mandibular gland compounds of virgin males and females

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Abstract. The secretion from the mandibular glands of males of the leaf-cutting ant *Atta sexdens rubropilosa* is responsible for the reaction of workers outside the nest at the time of sexual swarming. Workers respond with excitability and aggression when presented with the natural mixture of 4-methyl-3-heptanol and 4-methyl-3-heptanone, which is contained in the secretion of the male mandibular glands. Workers respond quickly to fractional amounts of one male equivalent. 4-Methyl-3-heptanone, from the virgin female mandibular glands causes much less response in workers, whereas an equimolar mixture of male and female pheromones gives a still less clear response. The male pheromone plays the most important part in the communication of workers outside the nest at this time.

Key words. Aggression, excitation, leaf-cutting ants, 4-methyl-3-heptanol, 4-methyl-3-heptanone, pheromone, virgin males, virgin females, worker behaviour.

Introduction

Immediately before the nuptial flights of leaf-cutting ants, it is common to observe great excitement and aggressiveness in the workers on the surface near the nests, in contrast to other times of the year. This behaviour is observed in several leaf-cutting ant species (Autuori, 1941; Moser, 1967; Mariconi, 1970; Moser *et al.*, 2004). It is suggested that the behaviour is a response to male mandibular gland secretions, and leads to protection of the sexual forms (virgin males and females) during swarming (Fowler, 1982). It is also claimed that the response helps workers to deter recently mated queens that might try to establish new nests too close to the established colony (Fowler, 1982; Vander Meer & Alonso, 1998).

The first study of mandibular glands of *Atta sexdens rubropilosa* worker identified citral (the equilibrium mixture of geranial and neral) and described it as a warning or frightening mixture (Butenandt *et al.*, 1959). Later studies showed that smaller workers produce 4-methyl-3-heptanone, whereas large workers and soldiers produce a mixture dominated by citral (do Nascimento *et al.*, 1993). The mandibular glands of virgin and mated queens essentially contain only 4-methyl-3-heptanone (5 μg per individual in virgin queens, 9 μg in mated queens). Unmated males produce a mixture of 4-methyl-3-heptanone and 4-methyl-3-heptanol in approximately equal proportions (11 μg per individual) (do Nascimento *et al.*, 1993). Mated males cease to contain the secretion (do Nascimento *et al.*, 1993).

4-Methyl-3-heptanone has been identified in the mandibular glands of several other species of *Atta*, namely *Atta bisphaerica*, *Atta capiguara*, *Atta cephalotes*, *Atta laevigata*, *Atta robusta* and *Atta texana* (Blum *et al.*, 1968; Riley *et al.*, 1974; Hernández *et al.*, 1999; Hughes *et al.*, 2001a; Hernández *et al.*, 2002). This compound also causes attraction in the grass-cutting ants *A. bisphaerica* and *A. capiguara* (Hughes *et al.*, 2001b).

Subsequently, the chirality of 4-methyl-3-heptanone and 4-methyl-3-heptanol from virgin males and females was determined, and (*S*)-(+)-4-methyl-3-heptanone essentially comprises the only material in the mandibular glands of

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Table 1. Intensity of reaction of workers of *Atta sexdens rubropilosa* to synthetic male mandibular pheromone (equal quantities of racemic 4-methyl-3-heptanone and racemic 4-methyl-3-heptanol, containing the stated amounts of the active chiral forms).

		Response intensity (%)	
Male equivalents	Workers responding, mean ± SD (%)	Type 2	Type 1
0.025	52.5 ± 10.8[b]	32.5[a]	20[d]
0.05	60 ± 5.8[b]	25[a]	35[c]
0.10	62.5 ± 2.5[b]	27.5[a]	35[c]
0.25	82.5 ± 4.3[a]	25[a]	57.5[b]
0.50	93.7 ± 9.5[a]	20[a]	73.7[a]
Control	5 ± 5.8[c]	5[b]	0[e]

Response intensity: type 1, workers turning towards source and opening and closing mandibles; type 2, same as type 1 with workers running excitedly over the test area. Means followed by the same superscript letter within a column do not differ among themselves by Tukey's test ($P = 0.05$).

mated and unmated queens. In males, it is a mixture of (3*S*,4*S*)-4-methyl-3-heptanol and *(*3*R*,4*S*)-4-methyl-3-heptanol, together with (*S*)-(+)-4-methyl-3-heptanone (do Nascimento *et al.*, 1997).

Moser *et al.* (1968) in *A. texana*, and Riley *et al.* (1974) in *A. cephalotes*, showed that (*S*)-(+)-4-methyl-3-heptanone is the only pheromonally active compound in these species and that it is unaffected by the presence of inactive (*R*)-(–)-4-methyl-3-heptanone. Other compounds identified in mandibular glands are inactive in behavioural assays. Little attention has been given to pheromones in sexuals, other than with respect to sexual attraction or copulation. The present study were therefore conducted with the more readily available racemic forms of 4-methyl-3-heptanone and 4-methyl-3-heptanol to observe the effects of male and female mandibular pheromones on workers of *A. sexdens rubropilosa.*

Materials and methods

Tests were carried out on two nests of *A. sexdens rubropilosa* that were approximately 3 years old, and contained 4–6 L of fungus. They were kept on a wooden foraging area (100 × 50 cm) at 25 ± 2 °C and 75 ± 5% RH under an LD 12 : 12 h photoperiod. Bioassays were conducted in a small arena (12 × 12 cm with a wall 6 cm high around it), which was placed randomly on the feeding table to isolate ten workers of different sizes for approximately 15 min before the beginning of each test. For the test, a solution containing the compound or mixture to be tested was placed on a piece of filter paper (2 cm in diameter) with a microsyringe, the solvent was allowed to evaporate, and the piece of filter paper placed in the centre of the arena. For a control, a piece of filter paper treated only with the solvent was used. The number of workers that responded to the stimulus, as well as the intensity of the response was quantified, according to the modified method of Fowler (1982). The percentage response of workers was obtained from the number of workers that gave a clear alarm or aggressive reaction to the stimulus. The response was characterized by two types: in a type 1 response, the workers opened and closed their mandibles in the direction of, or on top of, the stimulus. In a type 2 response, the workers additionally performed sharp body movements and ran excitably and aggressively in a direction away from the stimulus, over the test area. Each test was independently observed and scored by two individuals. To diminish the habituation effect, four replicate experiments were performed for each concentration, at intervals of at least 4 h, during 3 consecutive days. After this period, there was an interval of 3–5 days before the tests were repeated. Both nests were tested in the same way and the results were combined. The experiments were conducted in a completely randomized design. To determine the possible response of workers to different

Table 2. Intensity of reaction of workers of *Atta sexdens rubropilosa* to synthetic virgin female mandibular pheromone (racemic 4-methyl-3-heptanone containing the stated amounts of the active chiral form).

		Response intensity (%)	
Female equivalents	Workers responding, mean ± SD (%)	Type 2	Type 1
0.025	40 ± 10[b]	25[a,b]	15[a,b]
0.05	65 ± 12.9[a]	40[a]	25[a,b]
0.10	57.5 ± 8.7[a]	32.5[a]	25[a,b]
0.25	67.5 ± 11.9[a]	30[a]	37.5[a]
0.50	61.2 ± 13.1[a]	29[a]	32.5[a]
Control	7.5 ± 0.5[c]	7.5[b]	0[b]

Response intensity: type 1, workers turning towards source and opening and closing mandibles; type 2, same as type 1 with workers running excitedly over the test area. Means followed by the same superscript letter within a column do not differ among themselves by Tukey's test ($P = 0.05$).

Table 3. Intensity of reaction of workers of *Atta sexdens rubropilosa* to a combination of synthetic male mandibular pheromone (equal quantities of racemic 4-methyl-3-heptanone and racemic 4-methyl-3-heptanol), and female mandibular pheromone (racemic 4-methyl-3-heptanone) all containing the stated amounts of the active chiral form.

		Response intensity (%)		
Male equivalents	Female equivalents	Workers responding, mean ± SD (%)	Type 2	Type 1
0.10	0.10	81.2 ± 13.1[a,b]	27.5[a,b]	53.7[a,b]
0.25	0.10	96.2 ± 4.8[a]	36.2[a]	60[a]
0.50	0.10	67.5 ± 5[b,c]	32.5[a]	35[a,b]
0.10	0.25	80 ± 14.1[a,b]	40[a]	40[a,b]
0.25	0.25	70 ± 17.3[b]	30[a,b]	40[a,b]
0.50	0.25	66.7 ± 11.5[b,c]	36[a]	30.7[b,c]
0.10	0.50	45 ± 5.8[d]	30[a,b]	15[b,c]
0.25	0.50	32 ± 25.2[d,e]	22[a,b]	10[b,c]
0.50	0.50	20 ± 8.2[d,e]	15[a,b]	5[c]
Control	–	5 ± 5[e]	5[b]	0[c]

Response intensity: type 1, workers turning towards source and opening and closing mandibles; type 2, same as type 1 with workers running excitedly over the test area. Means followed by the same superscript letter within a column do not differ among themselves by Tukey's test ($P = 0.05$).

synthetic mandibular gland compounds of virgin males and females, the data for behaviour response and intensity (types 1 and 2) were submitted to analysis of variance, and the means were compared by Tukey's test ($P < 0.05$).

To determine the isomer composition of the synthetic 4-methyl-3-heptanol, a sample was chromatographed on a fused silica column (25 m × 0.25 mm) coated with Hydrodex β-6-TBDM (Macherey & Nagel, Germany) with temperature initially at 6 °C for 3 min, then programmed to 190 °C at 2 °C min^{-1}.

The synthetic 4-methyl-3-heptanol was shown to comprise 40.5% (3*S*,4*S*) and (3*R*,4*R*) isomers and 59.5% (3*S*,4*R*) and (3*R*,4*S*) isomers, whereas the 4-methyl-3-heptanol of male *A. s. ribropilosa* mandibular glands consists of equal proportions of (3*S*,4*S*) and (3*R*,4*S*) isomers (do Nascimento *et al.*, 1997). Therefore, in 100 ng of synthetic mixture, there are 20 ng of (3*S*,4*S*) isomer and 30 ng of (3*R*,4*S*) isomer. Although this differs from the natural ratio, we have made the approximation that 100 ng of synthetic mixture contains 50 ng of natural pheromone.

A solution of synthetic 4-methyl-3-heptanol (>99%, mixture of enantiomers, Aldrich, U.K) was prepared in hexane (HPLC grade, Fisher, U.K.) to give a concentration of 10 μg in 10 μL, which represents 500 ng μL^{-1} of the active mixture of (3*S*,4*S*)-4-methyl-3-heptanol and (3*R*,4*S*)-4-methyl-3-heptanol. Racemic 4-methyl-3-heptanone was produced by chromic acid oxidation of the corresponding alcohol and determined to be 99% pure by gas chromatography. This too was prepared as a solution (10 μg in 10 μL) in hexane, so that it contained 500 ng μL^{-1} of the active form (*S*)-(+)-4-methyl-3-heptanone. Further dilutions were made to represent fractional male and female equivalents for behavioural tests.

Results and discussion

When a stimulus corresponding to as little as 2.5% of a male mandibular gland contents was presented to a group of ten workers, half of them responded by turning towards the source and opening their mandibles (type 1 response) and 40% of those responding began to run excitedly about the arena (Table 1). Increasingly larger doses gave correspondingly stronger reactions that were significantly different when Tukey's test was applied (Table 1). At half the equivalent of the content of a male mandibular gland, 70% of the workers showed the intense response of type 2, running about the arena with their mandibles open. Only 5% of workers showed any response to the control papers. The results obtained clearly justify the use of the racemic mixture.

When the virgin female pheromone was similarly tested, the number of workers responding was less, and the number did not increase regularly with increasing doses (Table 2). An increase was noted only with the lower dose. The variation in response of individual workers was greater, and there was less agitation and running about.

When the male and female pheromones were combined in different proportions, the greatest and most aggressive response was with the lowest proportion of female pheromone (Table 3). Variation in response of individual workers was greater, and the excitability of workers decreased as the proportion of female pheromone increased.

Workers show a clear response to the male mandibular pheromone, and a closer dose–response relationship than is evident with either the female pheromone alone or with combined male and female pheromones. This confirms the observations of Fowler (1982) that the male mandibular gland pheromone should have an important role in the aggressive and excitable response of workers at the time of swarming. Moreover, earlier experiments demonstrate that the male mandibular glands show more secretion before mating than after mating (do Nascimento *et al.*, 1993), whereas the opposite is true for females, suggesting that female secretion is more important in some other function after mating.

That workers respond promptly to the lowest concentration of synthetic pheromone tested (0.025 equivalents of an average male sexual) indicates the important role of these

volatiles in communication for the species. The progressive increase in amount of 4-methyl-3-heptanone promotes atypical behaviour, such as crawling, momentary loss of movement and repellence. We conclude that the aggressive and excitable behaviour of workers close to the nest entrances before the nuptial flight is caused largely by discharge of the equimolar mixture of 4-methyl-3-heptanone and 4-methyl-3-heptanol from the mandibular glands of the unmated males present.

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