The Journal of Experimental Biology 211, 1109-1113
Published by The Company of Biologists 2008
doi:10.1242/jeb.008508

The mandible opening response: quantifying aggression elicited by chemical cues in ants

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Accepted 31 December 2007

SUMMARY

Social insects have evolved efficient recognition systems guaranteeing social cohesion and protection from enemies. To defend their territories and threaten non-nestmate intruders, ants open their mandibles as a first aggressive display. Albeit chemical cues play a major role in discrimination between nestmates and non-nestmates, classical bioassays based on aggressive behaviour were not particularly effective in disentangling chemical perception and behavioural components of nestmate recognition by means of categorical variables. We therefore developed a novel bioassay that accurately isolates chemical perception from other cues. We studied four ant species: *Camponotus herculeanus*, *C. vagus*, *Formica rufibarbis* and *F. cunicularia*. Chemical analyses of cuticular extracts of workers of these four species showed that they varied in the number and identity of compounds and that species of the same genus have more similar profiles. The antennae of harnessed ants were touched with a glass rod coated with the cuticular extract of (a) nestmates, (b) non-nestmates of the same species, (c) another species of the same genus and (d) a species of a different genus. The mandible opening response (MOR) was recorded as the aggressive response. In all assayed species, MOR significantly differed among stimuli, being weakest towards nestmate odour and strongest towards odours originating from ants of a different genus. We thus introduce here a new procedure suitable for studying the chemical basis of aggression in ants.

Key words: ants, aggression, mandible opening, nestmate recognition, chemical cues, cuticular hydrocarbons.

INTRODUCTION

Social insects are typically characterised by efficient recognition systems guaranteeing social cohesion and protection against robbery and parasitism from outside. Ant colonies, in particular, often have well-defined territorial boundaries which are aggressively defended against intruders of the same or different species (Hölldobler and Wilson, 1990). Behavioural assays in the form of aggression tests have been extensively used in a variety of ant species to study mechanisms underlying nestmate recognition (cf. Carlin and Hölldobler, 1986) as well as the loss of it [e.g. in supercolonies of invasive species (cf. Holway et al., 1998)]. These aggression tests try to reproduce, in different ways, the situation of encounters between ants. For example, an intruder introduced into a laboratory colony (Stuart, 1987); a group of ants (Errard et al., 2006) or two ants confronting each other in a neutral arena (d'Ettorre et al., 2000); or immobilised ants only allowed to move their head parts whilst facing each other (Lucas et al., 2005; Leonhardt et al., 2007). The level of aggression has been usually quantified by measuring the frequency and/or duration of different behaviours constituting a scale of aggressive displays ranging, for instance, from mutual tolerance (casual antennal contact) overt threat (opening of the mandibles) to overt attack (biting and flexing the gaster to spray formic acid or to sting). Overt attack is typically preceded by threat display in the form of mandible opening (Carlin and Hölldobler, 1986).

Many ants and other social insects discriminate between nestmates and non-nestmates by means of chemically perceiving the blend of hydrocarbons present on their cuticle, and there is an extensive literature on the role of cuticular hydrocarbons in ants (e.g. Bonavita-Cougourdan et al., 1987; Lenoir et al., 1999; Hefetz, 2007). The level of aggression towards an intruder may differ according to the similarity between the cuticular chemical profile of nestmates and that of the encountered individual (Lenoir et al., 1999). The aggression tests cited above do not allow an accurate assessment of the effect of chemical perception itself on aggression, since behavioural and/or visual cues may help the experimental ant to recognise other individuals. A previous attempt to separate the chemical component was made by Lucas et al. (Lucas et al., 2005): immobilised workers of the ant *Pachycondyla subversa* were presented with pieces of filter paper that had absorbed different mixtures of cuticular hydrocarbons and the duration of different behavioural responses was measured from video recordings. This was an interesting but quite laborious experimental procedure; the quantitative measurements were relatively difficult to standardise and possibly subject to an effect of the observer during the fine-graded analysis of the different aggressive displays (the variable measured were not categorical).

Procedures for specifically quantifying the individual response to a chemical cue have already been developed and successfully applied in honey bees. A typical response easy to quantify is the proboscis extension response (PER), an appetitive response exhibited by harnessed, hungry bees when their antennae are touched with sucrose solution (Kuwabara, 1957; Takeda, 1961). In an aversive context, the response that can be quantified is the sting extension response (SER), a defensive response exhibited by harnessed bees placed on a metallic holder through which an electric shock is applied (Núñez et al., 1983; Núñez et al., 1997). Also, PER and SER have been successfully combined to study, for instance, the modifications of the motivational state of the bee resulting from the exposure of the animals to alarm pheromones (Balderrama et al., 2002); the existence of genetic differences between individuals in their response

threshold (Page and Erber, 2002) and associative learning (Vergoz et al., 2007).

These procedures, which are repeatable and relatively simple to assay (because they give a 'yes or no' response), could be set as the standard for quantification of the response to stimulation, and have opened new avenues for research in learning and memory (reviewed by Giurfa, 2003; Menzel and Giurfa, 2006). It would be particularly interesting to perform analogous studies using ants – the most advanced among social insects – as models. Thus, the aim of the present study was to develop an accurate, simple and replicable procedure giving a categorical variable to measure the effect of chemical perception on aggression and to uncouple chemical perception from any other perceptual input. We took advantage of the opening of mandibles exhibited by ants as an aggressive display and studied how this display varies in harnessed ants stimulated with a chemical stimulus. We thus assessed the mandible opening response (MOR) as a measure of the aggression level in individual workers of *Camponotus herculeanus* Linnaeus, *C. vagus* Scopoli, *Formica rufibarbis* Fabricius and *F. cunicularia* Latreille. These species have been chosen because *Formica cunicularia* and *Formica rufibarbis* are closely related species that can live in sympatry and thus compete for exactly the same resources. They belong to the *Servifomica* group and can both be used as hosts by the same social parasites [e.g. *Polyergus rufescens* (d'Ettorre et al., 2002)]. *Camponotus vagus* and *C. herculeanus* are congeneric but allopatric and with different ecology (the first nests underground and the second in wood). Moreover, *C. vagus* and *F. cunicularia* are sympatric at our collecting site and possibly compete for the same resources, they both have underground nests that can be very spatially close, and their foraging territories overlap (personal observation).

We expected that the stereotyped MOR would differ according to the extracts presented to the experimental individual: extracts from its own nestmates or from non-nestmates of different categories (same genus, different genus), and that non-nestmates strongly differing in cuticular hydrocarbon profiles would be easier to identify, thus eliciting more aggression.

MATERIALS AND METHODS

Study organisms

Workers of two colonies of each ant species were used, both as test individuals and to prepare cuticular extracts. Colonies of *Camponotus herculeanus* were collected in Denmark (Laesoe); colonies of *Camponotus vagus* and *Formica cunicularia* in Italy (Apennines near Bologna); colonies of *Formica rufibarbis* were collected in Germany (Regensburg). They were brought to Copenhagen, Denmark, and kept under standardized laboratory conditions (24°C; 12 h:12 h L:D photoperiod).

Chemical analyses

To verify that the cuticular profile of the species involved in the study were indeed different, we analysed their cuticular hydrocarbons by gas chromatography and mass spectrometry (GC–MS). Cuticular hydrocarbons were extracted by washing individual ants in 200 μl of pentane for 10 min. After evaporation of the solvent, the extracts were diluted in 20 μl of pentane. In total, 40 extracts were prepared: five extracts from each colony of all the species involved in the experiment. Samples (2 μl) of each extract were injected into an Agilent Technologies 6890N gas chromatograph equipped with a capillary column (HP5MS 30 m×250 μm×0.25 μm). The injector was a split-splitless type, and the carrying gas was helium at 1 ml min^{-1}. The initial temperature was 70°C and was increased at 30°C min^{-1} to 200°C, then from 200°C to 310°C at 3°C min^{-1}, where it was held for 5 min. The gas chromatograph was coupled with a HS 5375 Agilent Technologies Mass Spectrometer using 70 eV electron impact ionization. Compounds were identified on the basis of their mass spectra, as well as by comparison with standards and published spectra (e.g. Bonavita-Cougourdan et al., 1991).

Experimental subjects

Individual worker ants were taken from inside their colony, in order to be sure they were in contact with their own colony odour. They were put into small glass vials (about 15 ml) and cooled on ice for 10 min, or until they stopped moving, and harnessed in an ant holder only allowing them to move their antennae and mouth parts. The ant holder consisted in an inverted 0.2 ml Eppendorf standard micro test tube, whose apex was cut off. The ant's head was passed through the apical hole of the tube and then fixed with an adhesive tape stuck behind the ant's neck (collum) pushing the head to the wall of the tube, leaving the mouthparts on the exterior side of the tube wall (Fig. 1). The ants were left in a quiet place undisturbed for 2 h in order to let them recover from the anaesthesia and habituate to the harness. After resting, the individuals that could actively move their antennae and mandibles (on average more than 90% of the harnessed individuals) were used for the tests.

Chemical stimuli

Cuticular extracts were used to stimulate the antennae of experimental ants. Extracts were prepared as described above under Chemical analyses, but using five workers per extract. The solvent was allowed to evaporate and the extracts were diluted in 50 μl of pentane. For each extract, 10 μm (containing on average the quantity corresponding to the extraction from one ant), were poured on the tip of a Pasteur pipette (hereafter referred to as the stimulation pipette) using a Hamilton syringe. The pipette was held with its tip downwards, thus keeping the extract around the outer part, up to 3 mm from the tip, until the pentane completely evaporated.

To quantify aggression in the four ant species (*C. herculeanus*, *C. vagus*, *F. rufibarbis* and *F. cunicularia*) five different types of stimulation pipettes were prepared, obtained from: (1) solvent alone (SOL); (2) extract of nestmate ants (NM); (3) extract of non-nestmate ants of the same species (NNM); (4) extract of ants of another species of the same genus (SG); (5) extract of ants of a different genus (DG). For *C. herculeanus*, SG was *C. vagus* and DG was *F. cunicularia*. For *C. vagus*, SG was *C. herculeanus* and DG was *F. cunicularia*. For *F. rufibarbis*, SG was *F. cunicularia* and DG was *C. vagus*. For *F. cunicularia*, SG was *F. rufibarbis* and DG was *C.*

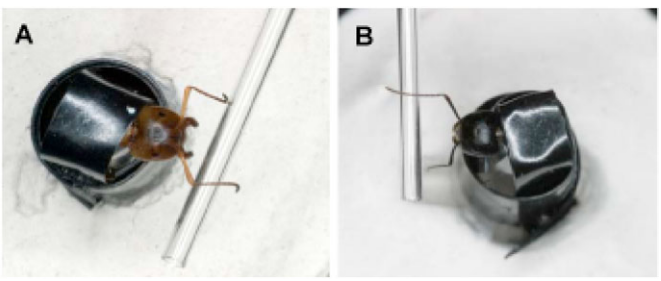

Fig. 1. Experimental design: ants were harnessed and could only move their antennae and mouthparts. (A) When stimulated with non-nestmate extract, the ant opens its mandibles showing aggression. (B) When stimulated with nestmate extract, the ant keeps its mandibles closed.

vagus. DG species such as *C. vagus* and *F. cunicularia* can live in sympatry and thus compete for the same resources (territories, nest sites).

A random sample (N=13) of pipettes used for stimulation were analysed by injecting a pentane wash into the GC–MS after use in the experiments. They all proved to have the pure initial compounds and demonstrated that no contamination had occurred during the experiments.

Experimental procedure

Each test was composed of five trials (five different stimuli). Each trial lasted 1 min and involved presenting one stimulus at a time to each test ant. One individual was placed under a stereomicroscope (Leica Wild M3B; oculars: Wild 445111 10×/21B; objectives: 6.4×). After 25 s, to allow habituation to the new context, the antennae were gently touched for 5 s with the tip of one of the stimulation pipettes. After another 25–30 s the individual was returned to its resting place. The inter-trial interval was 10 min to avoid any possible saturation of the antennal receptors. After that, the individual was set under the stereomicroscope again to be presented with the next stimulus. The procedure was repeated for all the five stimuli and the order of presentation was randomised. When the ant widely opened its mandibles, i.e. displacing them from their resting position, as the antenna was touched with the stimulation pipette, the response was noted as 1 (Fig. 1A), otherwise it was noted as 0 (Fig. 1B). The number of replicates (ants tested) was 25 individuals for each of the four species studied.

Statistics

We used two-way ANOVA to compare among the four assayed species, the number of ants opening their mandibles on their antennae being touched with each pipette tip. Although parametric ANOVA is not usually recommended in case of dichotomous data (1 *vs* 0), such as those of our MOR, Monte Carlo studies have shown that it is suitable under certain conditions, i.e. the proportion of responses in the smaller response category is at least 0.2 and there are at least 20 degrees of freedom for error (Lunney, 1970), which was the case in our study. This analysis is usually applied in studies quantifying PER in honey bees, whose data are also dichotomous [e.g. olfaction (Deisig et al., 2003; Guerrieri et al., 2005); gustation (De Brito-Sánchez et al., 2005); tactile stimulation (Giurfa and Malun, 2004)]. *Post-hoc* analyses were performed by means of Scheffé's contrasts.

After identification of the cuticular hydrocarbons by GC–MS, we quantified the presence or absence of hydrocarbons in the cuticular profiles of the four ant species. We counted the number of compounds that were not shared by two chemical profiles within a pair and we performed all possible pair-wise comparisons (i.e. *C. vagus vs C. herculeanus*, *C. vagus vs F. cunicularia*, *C. vagus vs F. rufibarbis*, *C. herculeanus vs F. cunicularia*, *C. herculeanus vs F. rufibarbis* and *F. cunicularia vs F. rufibarbis*). Thus, we could construct a matrix with these values and quantify similarity among profiles by calculating Euclidian distances and using Ward's method. The shorter the distance between two profiles, the greater the similarity.

RESULTS

Chemical analyses showed that the cuticular profiles of the four ant species involved in this study – *Camponotus herculeanus*, *C. vagus*, *Formica rufibarbis* and *F. cunicularia* – were qualitatively different among them, both in the number and the identity of the compounds (Fig. 2). However, the same compounds were consistently present on all workers of the same species, as is usually observed in ants (cf. Bonavita-Cougourdan et al., 1987; Lenoir et al., 1999; Hefetz, 2007). The observed differences among the cuticular profiles justify their use in our bioassay. The Euclidian distances measured showed that chemical profiles of species of the same genus are more similar than profiles of species belonging to different genera (Fig. 3).

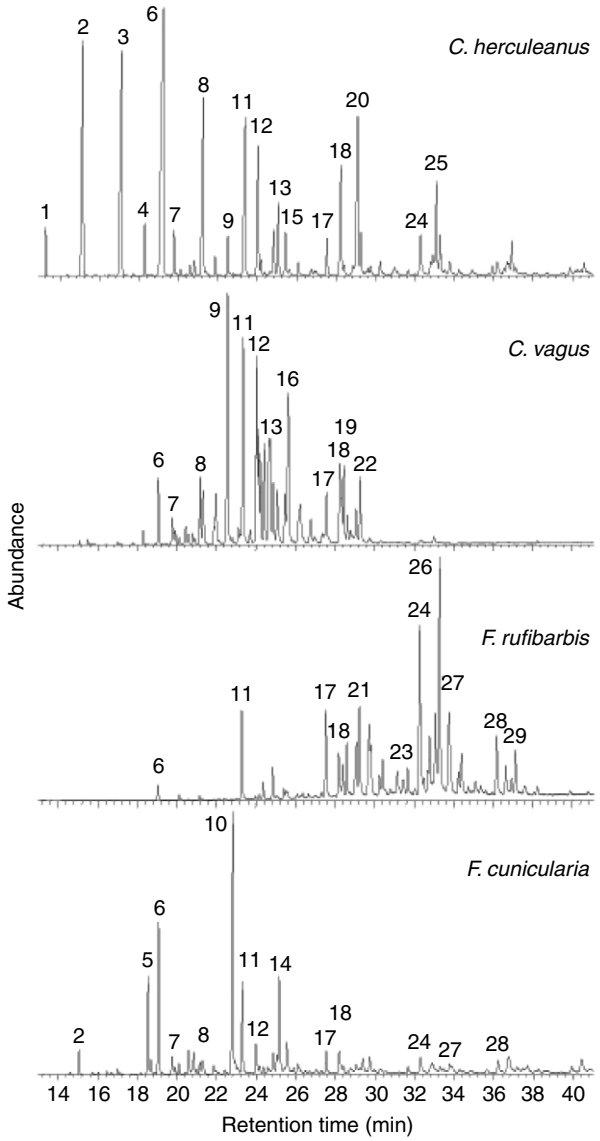

Fig. 2. GC–MS profiles of the cuticular hydrocarbons of the four ant species used in the experiments. Some of the identified peaks are indicated as a reference: (1) *n*-C_{22}; (2) *n*-C_{23}; (3) *n*-C_{24}; (4) 2-meC_{24}; (5)$C_{25:1}$; (6) *n*-C_{25}; (7) 9- + 11-meC_{25}; (8) *n*-C_{26}; (9) 2-meC_{26}; (10) $C_{27:1}$; (11) *n*-C_{27}; (12) 11- + 13-meC_{27}; (13) 5-meC_{27}; (14) $C_{28:1}$; (15) *n*-C_{28}; (16) 10-meC_{28}; (17) *n*-C_{29}; (18) 11- + 13-meC_{29}; (19) 7-meC_{29}; (20) 7,13-dimeC_{29}; (21) 5,11-dimeC_{29}; (22) 12-meC_{30}; (23) *n*-C_{31}; (24) 13- + 15-meC_{31}; (25) 7-meC_{31}; (26) 5,13-dimeC_{31}; (27) 3,11-dimeC_{31}; (28) 15- + 17-meC_{33}; (29) 5-meC_{33}.

We compared the number of ants among four species opening the mandibles according to the stimulus presented in each test-trial. The two-way ANOVA yielded a highly significant stimulus effect ($F_{4,384}$=28.43; P<0.0001), but neither a significant species effect ($F_{3,96}$=1.03; P=0.38), nor a significant interaction between both effects ($F_{12,384}$=0.95; P=0.50). The general trend was that aggression (MOR) increased when the stimulus presented to the test ant differed the most from the test ant's cuticular extract (Fig. 4).

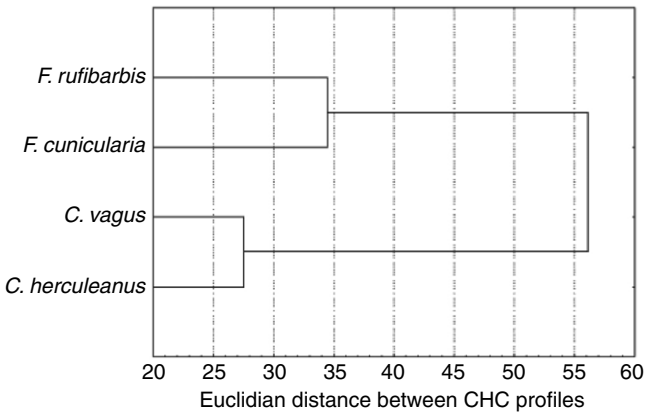

Fig. 3. Dendrogram based on Euclidian distances between cuticular hydrocarbon (CHC) profiles of the four ant species used in the experiments. Cuticular hydrocarbon profiles of species of the same genus are more similar (closer) than profiles of species of different genera. The shorter the distance between two profiles, the greater the similarity.

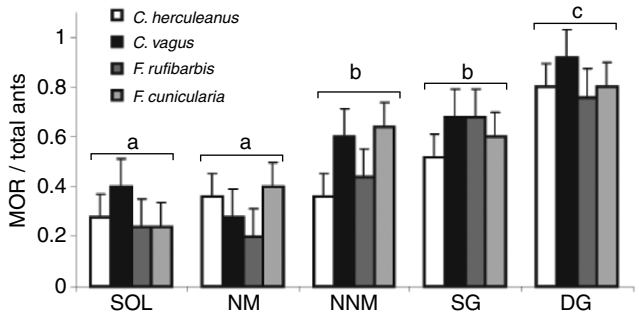

Fig. 4. Mandible opening response (MOR) mean (± s.e.m.) for each of the four assayed species. The responses significantly differed among stimuli ($F_{4,384}$=28.43; P<0.0001). Species effect ($F_{3,96}$=1.03; P=0.38) and interaction between both effects ($F_{12,384}$=0.95; P=0.50) were non-significant. Stimuli: solvent (SOL), or extracts from nestmate (NM); non-nestmate of the same species (NNM); different species of the same genus (SG); different genus (DG). Different letters indicate significant differences (Scheffé's *post-hoc* test, P<0.03 in all cases).

In particular, MOR towards DG was greater than towards all the other stimuli (Scheffé's *post-hoc* test, P<0.03 in all cases) and MOR towards SG and NNM were greater than MOR towards NM (P<0.03 in all cases). However, individual species might show slightly different responses. For instance, *C. herculeanus* did not significantly differentiate between NM and NNM, but followed the general trend towards SG and DG. For all species, there was no statistical difference between MOR elicited by the presentation of NM extracts and solvent alone.

DISCUSSION

We have developed a simple and accurate procedure for quantifying the effect of chemical stimulation on aggression in ants by using a categorical variable. This procedure is repeatable and comparable in different ant genera. We found that the mandible opening response (MOR) differed according to the stimulus presented to ants of the four different species used. All the species assayed showed the same trend, namely the MOR being greater as the presented cuticular hydrocarbon extract differed more from that of the experimental subject. Moreover, the response to non-nestmate extracts was greater than the response to nestmate extracts. The fact that neither the species effect nor the interaction between species and stimulus effects were statistically significant, suggests that the MOR is a suitable tool to study and quantify aggression due to chemical stimulation in ants in general, even if there might be slight differences in the response of individual species (e.g. *C. herculeanus*). Given that threat by mandible opening is a very common display, we expect that the MOR procedure can be generally applied to study the chemical bases of nestmate recognition and discrimination in ants, as well as to better understand the role of other chemicals (e.g. alarm pheromones).

Opening or not of the mandibles represents a conservative qualitative binomial variable that clearly indicates whether an aggressive response is elicited. It is expressed consistently enough to provide considerable statistical power. Indeed, we could apply the standard statistics for analysing PER and SER in honey bees to MOR data in ants. An advantage of using our MOR procedure is that it allows effective separation of the chemical component of the stimulation from behavioural cues by recording only the first aggressive display (mandible opening). Ants responded differently according to each chemical stimulus with which they were presented. This provides evidence that the possible level of stress provoked by harnessing conditions was not high enough to interfere with the ants' motivational states. The reaction to the stimulation pipette treated only with the solvent or with nestmate extracts served to control whether visual and tactile stimulations could be at the origin of MOR. Since these two stimuli elicited the lowest response level, we can safely assume that the differences in aggression were indeed due to differences in the origin of the chemical stimuli.

We can conclude that differences among ants' responses were due to differences among the chemical stimuli with which the ants were presented. Therefore, ants can be tested under these experimental conditions to study the effects of either a certain chemical blend or the effect of any particular chemical compound. Each substance constituting the cuticular extract could be presented to the antennae separately or in a particular mixture, thus allowing future research to analyse which substances plays a major role in nestmate recognition.

The MOR procedure could be also used to investigate whether a previous presentation of a neutral chemical stimulus can be associated with a subsequent presentation of a non-nestmate extract acting as an unconditioned stimulus. As well, it will be interesting to study the role of biogenic amines in the modulation of MOR and any possible association between MOR and other stimuli, as has been successfully done in honey bees by using PER and SER (Giurfa, 2006; Giurfa, 2007; Vergoz et al., 2007). If any association between MOR and other stimuli can be experimentally established, it will be possible to use MOR to study learning and memory in ants, similarly to the plethora of studies on odour and taste conditioning that have been performed in honey bees using PER (cf. Menzel and Giurfa, 2006), and those that will follow in the near future using SER (cf. Vergoz et al., 2007; Giurfa, 2007).

This work was financed by the Marie Curie Excellence Grant CODICES (MEXT-CT-2004-014202) assigned to P.dE. We thank the staff of the *Centre for Social Evolution*, University of Copenhagen, for the stimulating working environment. The images in Fig. 1 were taken by David R. Nash. We also thank Sophie Armitage, Koos Boomsma, Stéphanie Dreier, Martin Giurfa, Joaquín Hortal, David Hughes and Jelle van Zweden for helpful comments on the manuscript. The species were identified by Bernhard Seifert, Staatliches Museum fuer Naturkunde Goerlitz, Germany. Two anonymous reviewers gave valuable suggestions that improved the manuscript.

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