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SHORT COMMUNICATION

Sensing the intruder: a quantitative threshold for recognition cues perception in honeybees

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Abstract The ability to discriminate among nestmates and non-nestmate is essential to defend social insect colonies from intruders. Over the years, nestmate recognition has been extensively studied in the honeybee *Apis mellifera*; nevertheless, the quantitative perceptual aspects at the basis of the recognition system represent an unexplored subject in this species. To test the existence of a cuticular hydrocarbons' quantitative perception threshold for nestmate recognition cues, we conducted behavioural assays by presenting different amounts of a foreign forager's chemical profile to honeybees at the entrance of their colonies. We found an increase in the explorative and aggressive responses as the amount of cues increased based on a threshold mechanism, highlighting the importance of the quantitative perceptual features for the recognition processes in *A. mellifera.*

Keywords Honeybee · Recognition · Perception · Cuticular hydrocarbons · Threshold

Introduction

Discrimination between group members and strangers represents a key feature of social life. The ability of recognizing potential intruders, who try to sneak into a colony to exploit its valuable resources, proves essential for the evolution, the integrity and the survivorship of the insect societies (Wilson and Hölldobler 2005). The importance of a sophisticated recognition system as the basis for an effective colony defence has been demonstrated in a lot of studies (for a review see van Zweden and D'Ettorre 2010) showing the predominant role of semiochemicals, mainly cuticular hydrocarbons (CHCs), as cues for recognition (Howard and Blomquist 2005). Despite the considerable attention devoted to the subject, most of the work carried out so far focused on the qualitative aspects on the basis of the recognition process. Nestmate recognition in social insects has been deeply investigated in terms of differential importance of environmental versus heritable components and structural groups of compounds responsible for the 'inner template' used in deciding whether an individual is a nestmate or not (van Zweden and D'Ettorre 2010). On the contrary, very few studies have examined the quantitative perceptual features involved in the recognition process (Cini et al. 2009; Ichinose and Lenoir 2010). Recognition requires the perception of cues emitted by the encountered individual, in which cues are compared to the template of the receiver and their amount plays a fundamental role in perceiving and recognizing a potential intruder (Cini et al. 2009). The European honeybee *Apis mellifera* has been extensively used as a biological model for studying nestmate recognition and acceptance threshold in social insects (Breed 1983; Downs and Ratnieks 2000; Fröhlich et al. 2000; Couvillon et al. 2008); nevertheless, the existence of a quantitative perceptual threshold for nestmate recognition has been widely assumed and often taken for granted, even though direct tests have never been carried out in this species. Here we address such a topic, testing the existence of a quantitative CHC perception threshold in *A. mellifera* and, at the same time, quantifying the lower limit of nestmate recognition cues needed to elicit a behavioural reaction in this species.

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Materials and methods

To test the existence of a quantitative CHC detection threshold for nestmate recognition, we presented honeybee colonies (N=20) with different amounts of CHC extracts from foreign foragers: namely one eighth, one quarter, one half and the full extract, and a blank control represented by the solvent used for extraction. Foreign foragers (N=80) used for lure preparation (see below) were collected at the entrance of a colony from a different apiary brought to the lab and killed by freezing.

Lure preparation

CHC extracts (N=80) were obtained by washing each forager in 1 ml of pentane for 1 h. The extracts were then dried and re-suspended in 100 μl of pentane in the case of full extracts and the fractions of one half and one quarter. Lures were obtained by placing different amounts of re-suspended extracts on a square piece of clean filter paper with approximately 0.5 cm side. Total extract lures (N=20) were prepared by putting the entire volume of 100 μl on the filter paper. For one-half (N=20) and one-quarter (N=20) lures, the paper lures were covered with 50 and 25 μl respectively of the total extract. Finally for one-eighth extracts (N=20), the dried content of each vial was re-suspended in 200 μl of pentane and the lures were covered with 25 μl of the total extract. In this case we doubled the re-suspension volume because 12.5 μl out of 100 μl of solvent proved insufficient to cover the entire paper lure. Control lures were obtained by using 100 μl of the solvent only.

Chemical analyses

To verify if the subsequent dilutions of a full extract effectively provided fractions with a reduced amount of CHCs without altering the chemical profile of bees, we carried out preliminary gas chromatography–mass spectrometry (GC-MS) analyses. The forager extracts were obtained by washing each bee (N=5) in 1 ml of pentane for 1 h. The solvent was then evaporated under a nitrogen stream, each extract was re-suspended in 200 μl of pentane and the different fractions were prepared. Each fraction was then added with *n*-hexadecan-1-ol at a concentration of 10 ng/μl as the standard. Analyses of 1 μl of extract for each fraction were performed using a Hewlett Packard (Palo Alto, CA, USA) 5890A gas chromatograph coupled to an HP 5971 mass selective detector (using 70 eV electronic ionization source) following the standard procedure reported in Cappa et al. (2013). For each chromatogram, the areas of all the quantifiable peaks were summed and then compared to the area to obtain the total amount of CHCs in each fraction.

Behavioural assays

Lure presentations were carried out in the field at the beginning of July because the paucity of nectar at this time of the year prompts the bees to defend more strenuously the hive entrance (Downs and Ratnieks 2000). Each lure was set on a long stick and slowly brought nearby the entrance of the beehive. The lure was gently moved alongside the entrance contacting as many bees as possible in order to record their reactions. Each presentation lasted 1 min starting from the first interaction of a bee with the lure. To avoid sensibilization or habituation effects on the bees' response, all the colonies were presented with the five concentration lures in a random order, with a 15-min interval between two successive presentations. Assays were carried out on a single day, between 10:00 and 16:00, and each presentation was video-recorded. Both the operators responsible for presenting the lure and video recording the bees' response were blind to the presentation order. Colonies were presented with different sets of the five lures and every lure was presented only once. All video recordings were blind-watched in slow motion (0.25 s) independently by two viewers who registered the total number of explorative or aggressive contacts (i.e. antennations, darts, bites) of the bees towards the lure. Given the difference in the number of bees found at the entrance of each colony, we calculated a response index by dividing the total number of registered contacts for the average number of bees at the entrance (as the average between the total number of bees at the entrance respectively at the beginning and at the end of presentation). Behavioural data obtained from video were analysed with non-parametric Friedman test for multiple comparisons of paired data. Post hoc tests (Wilcoxon non-parametric tests using the Monte Carlo method) were used to assess if, and where, a significant difference existed between the pairs of treatments with a P value of less than α/number of comparisons (0.05/10=0.005). Statistical analyses were performed using the program SPSS 16.0 for Windows (SPSS Inc., Chicago, IL, USA).

Results

GC-MS analyses showed that subsequent dilutions effectively provided extract fractions with the expected amount of CHCs without altering the chemical profile (Fig. 1). The quantitative analyses showed that the amount of CHCs obtained with our method of extraction was on average of ~160 μg for the total extract (ranging from ~158 to ~162 μg) and on average of ~20 μg in the one-eighth fraction (ranging from ~19 to ~22 μg). The number of explorative and aggressive contacts differed between the treatments (Friedman test, χ^2=39.88, df=4, P<0.0001, N=20 colonies). The one-eighth extracts did not elicit a higher response than the controls (Z=-1.307, P=0.191), while the one quarter, the one half and the total

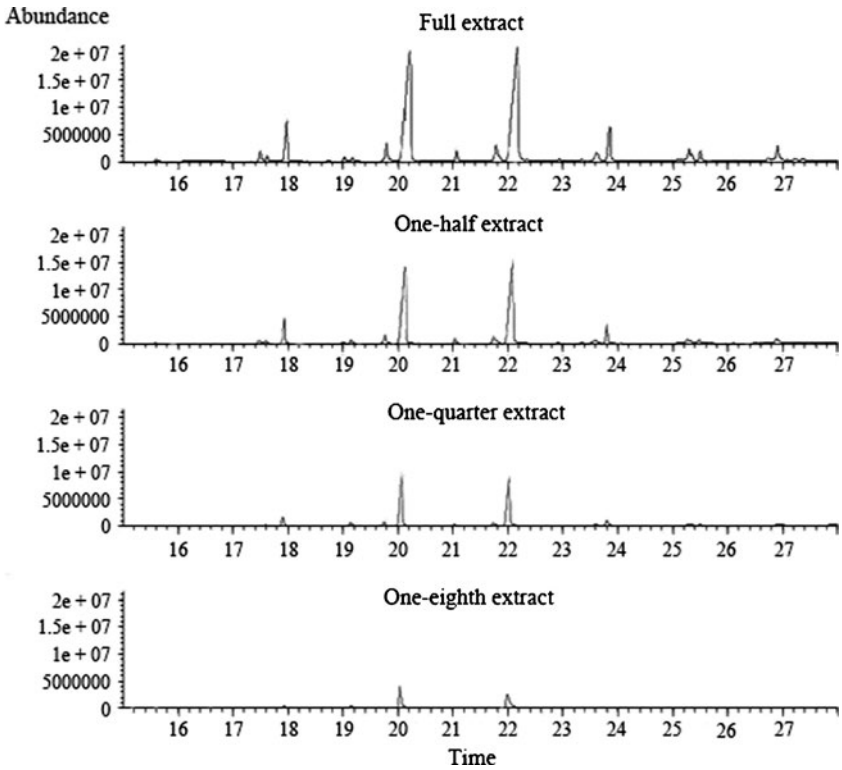

Fig 1 Chromatograms showing the different amounts of CHC extract from a forager beex used in our bioassays

amount extracts did (Z=−3.696, P<0.0001; Z=-3.771, P<0.0001; Z=-3.808, P<0.0001, respectively). There was no difference in the responses evoked by the one quarter, one half and the total extracts (1/4 vs. 1/2, Z=-1.643, P=0.100; 1/4 vs. total, Z=-1.232, P=0.218; 1/2 vs. total, Z=-0.672, P=0.502), while all of them differed significantly from the one-eighth forager equivalents in terms of elicited response (Z=-2.987, P=0.003; Z=-3.435, P=0.001; Z=-3.659, P<0.0001 respectively) (Fig. 2).

Discussion

Our results show that the different amounts of CHCs from a foreign forager elicit different levels of exploratory or aggressive response in honeybees at the entrance of a colony. The full amount significantly elicited stronger responses than the one-eighth fraction and the control, while fractions of, respectively, one half and one quarter of the total were treated as the full extract. Thus, our findings support the hypothesis of a threshold mechanism in the chemical recognition system of *A. mellifera*, with CHC perception occurring only above a certain amount of cues, in accordance with what previously found in *Polistes* wasps (Cini et al. 2009) and *Aphaenogaster* ants (Ichinose and Lenoir 2010). Interestingly, the one-eighth forager equivalent, corresponding to approximately 20 μg of CHCs, seems relatively high if compared to the sensitivity to hydrocarbons found in other insects; however, the sensitivity of social insects to CHCs in terms of quantity is still a poorly understood subject (see Ichinose and Lenoir 2010). Fröhlich et al. (2001) used the proboscis extension response (PER) to test the ability of bees to discriminate between cuticular waxes from drones and workers performing different tasks, while Dani et al. (2005) studied the differential importance of cuticular alkanes and alkenes in honeybees' nestmate recognition through supplementation of naturally occurring CHCs on live

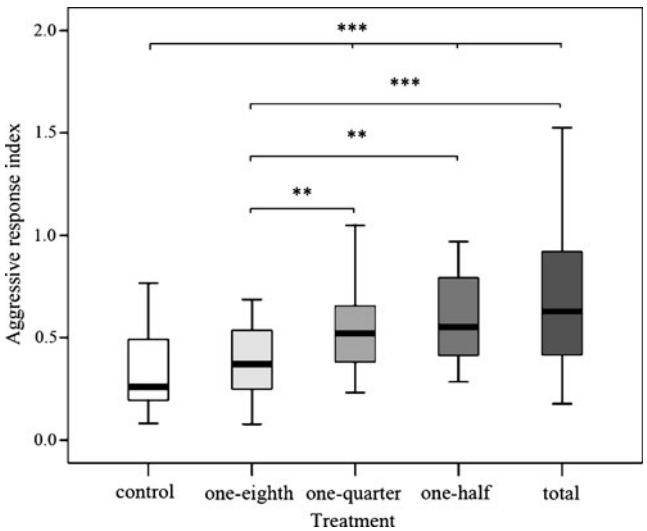

Fig 2 Behavioural responses of bees belonging to different colonies (N= 20) towards lures covered with different amounts of a foreign forager's CHCs. Bees increased their responses showing a threshold mechanism in the CHC perception. *Box plots* represent the medians (*thick horizontal lines*), the interquartile range (*boxes*) and the top and lowest quartiles (*horizontal lines*)**P<0.01; ***P<0.0001

foragers. In these cases, however, the quantity of CHCs used to treat each forager (100–200 μg, Fröhlich et al. 2001; 66.6 μg, Dani et al. 2005) was well above our one-eighth forager equivalent corresponding to our sensitivity threshold. More interestingly, Châline et al. (2005) also used PER conditioning paradigm to demonstrate that honeybees are able to recognize and learn different hydrocarbons, especially alkenes, when each compound is presented in a concentration of 20 μg, corresponding approximately to the quantity of CHCs in our one-eighth forager equivalent. A possible explanation for the fact that bees were able to respond to some of the presented compounds at such concentration, while they did not respond to our one-eighth forager equivalents, is that our fractions contained a mixture of the different CHCs found on a honeybee body and not a single compound. In our one-eighth fractions, the concentration of each single compound was probably lower than the 20 μg used by Châline et al. (2005) and thus undetectable for the honeybees. According to the 'chemical insignificance' hypothesis (Lenoir et al. 1999), intruders may exploit a quantitatively undetectable chemical profile to elude the colony defensive system. Such a deceptive strategy is adopted by some species of social parasites (Lenoir et al. 2001; Lorenzi et al. 2004), and it may also be exploited by other parasites and pathogens that alter the chemical profile of their hosts to make them undetectable in order to promote transmission among colonies. In facts, bees infected by the deformed wing virus (DWV) do not show the increase in the total amount of CHCs with age characteristic of healthy individuals, suggesting that immunostimulation induced by DWV may strongly alter existing biosynthesis or transport pathways and thereby shift cuticular chemical profiles of their hosts (Baracchi et al. 2012). Overall, our study fills a gap in the knowledge on the perception of chemical recognition cues in *A. mellifera*, focusing on the quantitative aspects and providing the direct evidence that the amount of semiochemicals plays a crucial role in the recognition processes of honeybees.

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