Learning and discrimination of cuticular hydrocarbons in a social insect

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Social insect cuticular hydrocarbon (CHC) mixtures are among the most complex chemical cues known and are important in nest-mate, caste and species recognition. Despite our growing knowledge of the nature of these cues, we have very little insight into how social insects actually perceive and discriminate among these chemicals. In this study, we use the newly developed technique of differential olfactory conditioning to pure, custom-designed synthetic colony odours to analyse signal discrimination in Argentine ants, Linepithema humile. Our results show that tri-methyl alkanes are more easily learned than single-methyl or straight-chain alkanes. In addition, we reveal that Argentine ants can discriminate between hydrocarbons with different branching patterns and the same chain length, but not always between hydrocarbons with the same branching patterns but different chain length. Our data thus show that biochemical characteristics influence those compounds that ants can discriminate between, and which thus potentially play a role in chemical signalling and nest-mate recognition.

Keywords: Nest-mate recognition; cuticular hydrocarbons; chemical communication

1. INTRODUCTION

Many animals signal using complex chemical mixtures that function only when perceived correctly by the intended receiver. Nevertheless, the primary focus of research into chemical communication has been the nature of the signal and the signaler rather than the receiver [1]. Social insects have some of the most sophisticated chemical communication systems, using numerous pheromones and chemical cues for interactions between colony members. Cuticular hydrocarbons (CHCs) play a central role in insect communication [2]. An insect’s CHC profile usually comprises a complex mixture of n-alkanes, methyl-branched alkanes and alkenes, and may contain over 100 different compounds [3].

We know surprisingly little about how ants perceive a CHC profile, despite its importance in maintaining colony cohesion. Recent experiments that manipulate the CHC profile with synthetic hydrocarbons [4–6] reveal that some hydrocarbons elicit aggression. However, the perception component in these experiments is entangled with the action component: a lack of aggression does not necessarily mean a failure to perceive this compound. Electrophysiological studies of antennal activity provide a measure of detection, but CHCs are non-volatile, insoluble in water and therefore difficult to record using conventional methods [7].

Classical conditioning and differential conditioning experiments offer promising approaches to studying the perception and discrimination of non-volatile compounds such as CHCs [8]. Often, a response, such as the proboscis extension response [9], is conditioned by associating an odour with a sucrose reward. We used differential olfactory conditioning to pure, custom-designed synthetic colony odours to illuminate signal learning and discrimination in Argentine ants, Linepithema humile. We employed the maxilla-labium extension conditioning response (MaLER; following [10]) to compare the learning ability of Argentine ants for three classes of compounds: straight-chain alkanes, single- and tri-methyl-branched alkanes. We used synthetic, custom-designed versions of methyl-branched hydrocarbons that are sufficient to trigger aggression among nest-mates [4,6]. We address two specific questions: Which classes of compounds are learned most easily, and thus most likely used in colony mate recognition? How do functional chemical characteristics, such as carbon-chain length and methyl branch location, influence chemical discrimination?

2. MATERIAL AND METHODS

(a) Study organism

We collected Argentine ants from Santa Clara County, CA, USA. We established artificial nests in Petri dishes lined with Fluon and containing a single nesting chamber. These nests were composed of two queens, brood and workers and were food-deprived for at least one week before the assays.

(b) Preparation of hydrocarbons

We tested different combinations of six custom-designed synthetic hydrocarbons [4,6]: three single-methyl-branched (15-MeC37, 17-MeC35 and 17-MeC37) and three tri-methyl-branched (3,13,17-triMeC33, 3,13,17-triMeC35 and 3,13,17-triMeC37) hydrocarbons. In all cases, mixtures of stereoisomers were used for the experiment. As a control, we used a straight-chain alkane (C36) (n-Hexatriacontane, Sigma, USA). We prepared 1 or 0.1 mg ml⁻¹ solutions in hexane (n-Hexane, Fisher Scientific, USA). Given the high viscosity of tri-methyl-branched CHCs, we used 0.1 mg ml⁻¹ solutions for all the pairs involving these compounds. We applied 10 µl of the hydrocarbon solution to the tip-end of a 1 µl glass capillary (1 µl Microcap, Drummond Scientific, USA) and evaporated the hexane at room temperature for 1 h. For each worker, we used the same capillaries for the duration of the trial.

(c) Experimental procedure

We employed MaLER (following Guerrieri & d’Ettorre [10]) to determine whether Argentine ants discriminate between compounds. We conducted differential olfactory conditioning assays in which one hydrocarbon was rewarded (the conditioned stimulus, CS+) and one hydrocarbon was unrewarded (CS−). We chilled the ants until motionless, then pushed them to the end of a P200 pipette tip until their entire head, including antennae, protruded from the tip. Workers were then kept in the pipette tip for an hour to habituate prior to the experiment. The horned ants were then presented with two hydrocarbons at 15 min intervals in the following sequence: −−−−−−−−−−−−−− . The plus (+) symbol represents the rewarded hydrocarbon (CS+), which was followed 3 s later by the sucrose reward. The minus (−) symbol represents the
unrewarded hydrocarbon (CS−). For a sucrose reward, we used a 20% w/w sucrose solution, which we presented to the ants on cotton. The hydrocarbons were presented by touching the antennae of the ant for 10 s, during which we recorded the presence of a MaLER.

We determined whether workers discriminate between different kinds of hydrocarbons by comparing their behaviour with the following combinations. (i) methyl-branched and straight-chain hydrocarbons: 17-MeC35 (CS+) versus C36 (CS2), and C36 (CS+) versus 17-MeC35 (CS−); (ii) hydrocarbons with the same methyl branching, but different chain lengths: 17-MeC37 (CS+) versus 17-MeC35 (CS−); 3,13,17-triMeC35 (CS+) versus 3,13,17-triMeC33 (CS−); and 3,13,17-triMeC37 (CS+) versus 3,13,17-triMeC33 (CS−); (iii) hydrocarbons with the same chain length, but different methyl branching: 15-MeC37 (CS+) versus 17-MeC37 (CS−), and 3,13,17-triMeC35 (CS+) versus 17-MeC35 (CS−); and (iv) hydrocarbons that differed in both chain length and methyl branching: 3,13,17-triMeC33 (CS+) versus 17-MeC35 (CS−).

Ants responding to the first CS− and/or CS+ were discarded from the analysis as their responses may be triggered by mechanical stimulus. Ants that did not respond to the sucrose reward at least four times were discarded. We used 633 ants for the bioassays but discarded 152 individuals (including ants showing spontaneous responses, positively responding less than four times to the CS−, dead and escaped ants).

### Statistical analyses
We counted the total number of responses for each hydrocarbon and tested whether ants preferred the CS+ to CS− using Wilcoxon-matched pairs signed-rank tests. To quantify the abilities of the ants to learn each hydrocarbon, we used a learning index [8], which is defined by the proportion of positive responses to the CS+ (out of six, as the response to the first CS+ is always negative). The learning index ranges between 0 and 1, with 1 representing perfect learning. To test for significant differences between learning indices for the different compound groups, we used Kruskal–Wallis tests, first to test for an overall significant difference, second to test for significant differences between particular compound groups. Because ants are less likely to learn differences in compounds which they cannot discriminate, we also conducted an analysis including only hydrocarbon pairs for which there was significant discrimination. To reduce the risk of type I error, we conducted Bonferroni corrections which maintained the experiment-wise error rate at α = 0.05.

### 3. RESULTS
We successfully employed the MaLER assay to test eight hydrocarbon pairs, using 481 Argentine ants, which responded significantly differently in five of these pairs (Wilcoxon-matched pairs signed-rank test, figure 1). There was a significant difference in the number of positive responses between the methyl-branched alkene 17-MeC35 and the straight-chain...
alkane C36 when the 17-MeC35 was the CS+, but this difference was not evident when the C36 was the CS+ (figure 1a,b), indicating that the ants were not able to perceive the C36. Ants discriminated between two single-methyl-branched hydrocarbons that have the same chain length but differ in the position of the methyl branch (17-MeC37 and 15-MeC37, figure 1d), but not between hydrocarbons that have the same methyl branch position but the chains differ in length by two carbons (17-MeC37 and 17-MeC35, figure 1c). Note that the first pair differs in the hydrocarbon tails by two carbon units on both ends, while the second pair differs only at one end. Similarly, the ants did not discriminate between tri-methyl-branched hydrocarbons that have the same methyl branch position, but chains differ by two carbons in length (5,13,17-triMeC33 and 5,13,17-triMeC35, figure 1e). However, the ants discriminated between tri-methyl hydrocarbons with the same methyl branching but differing by four carbons in length (5,13,17-triMeC33 and 5,13,17-triMeC37, figure 1f). Finally, ants discriminated between a tri-methyl-branched hydrocarbon and a single-branched hydrocarbon when both branching and chain length differed between the hydrocarbons (5,13,17-triMeC33 and 17-MeC35, figure 1f), and also when only the branching, but not the chain length was different (5,13,17-triMeC35 and 17-MeC37, figure 1g).

There were significant differences in the ability of ants to learn the different types of compounds when used as CS+, both when we included all hydrocarbon pairs (Kruskal–Wallis test \( p < 0.001 \), figure 2) and when we included only the hydrocarbon pairs for which there was significant discrimination (Kruskal–Wallis test \( p < 0.001 \)). Ants were significantly more successful at learning the tri-methyl hydrocarbons than the single-methyl or the straight-chain alkanes (in both comparisons including all hydrocarbon pairs \( p < 0.001 \), and in both comparisons only including hydrocarbon pairs for which there was significant discrimination \( p = 0.001 \), all of which survive Bonferroni correction for multiple comparisons). There was no significant difference in their capacity to learn the straight-chain alkane and the single-methyl alkane both including all hydrocarbon pairs \( p = 0.228 \) and only including hydrocarbon pairs for which there was significant discrimination \( (p = 0.146) \).

4. DISCUSSION

Although several studies reveal that methyl-branched alkanes or alkenes, but not \( n \)-alkanes, elicit aggression from colonymates [4,5,11,12], they cannot separate the perception component from the action component. We show that structurally more complex chemicals, such as tri-methyl-branched CHCs, are more easily learned by ants than comparatively simple compounds, such as single-methyl or straight-chain alkanes. These data suggest that more complex compounds are easier to detect than simple ones as the ability to learn odours will most probably be linked to their detection by the nervous system [8]. However, mixtures of stereoisomers were synthesized and used in the experiments, and there were necessarily more enantiomers for the tri-methyls (eight) than single-methyls (two). It could be that more complex compounds with more stereoisomers have greater delectability because the receptors have more molecular forms to perceive.

Not all hydrocarbons in the CHC profile are independently used as recognition cues by Argentine ants; workers discriminate between hydrocarbons with different branching patterns and the same chain length but not always between hydrocarbons with the same branching patterns but a different chain length. The nature of the difference between compounds is important: for example, methyl position plays a key role in allowing Argentine ants to discriminate between two hydrocarbons, with even slightly different methyl branch positions being distinguishable.

Studies investigating differences among CHC profiles typically use individual compound peaks obtained by gas chromatography (GC) as independent variables. Our data suggest that this use of individual compound peaks as independent variables is inappropriate. Ants discriminate between compounds that elute as a single GC peak (e.g. single-methyl hydrocarbons with the same chain length), and may also perceive one signal for hydrocarbons that elute as multiple GC peaks (hydrocarbons with different chain lengths but the same methyl branching). Clearly, compounds that are generalized should be treated as a single variable and co-eluting hydrocarbons that ants discriminate between should be treated as multiple variables. The latter requires techniques that allow complete separation of many of the hydrocarbons in the CHC profile, which are currently unavailable. However, insights into the ratios of the different hydrocarbons within peaks may possibly be obtained by dividing the peaks according to the ratios in which the diagnostic ions occur [13]. Nevertheless, investigations that focus on the capacity of the receiver to detect different compounds is likely to reveal more subtle intra- and intercolony differences than are currently known.

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