

RESEARCH ARTICLE

Chemical structure of odorants and perceptual similarity in ants

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SUMMARY

Animals are often immersed in a chemical world consisting of mixtures of many compounds rather than of single substances, and they constantly face the challenge of extracting relevant information out of the chemical landscape. To this purpose, the ability to discriminate among different stimuli with different valence is essential, but it is also important to be able to generalise, i.e. to treat different but similar stimuli as equivalent, as natural variation does not necessarily affect stimulus valence. Animals can thus extract regularities in their environment and make predictions, for instance about distribution of food resources. We studied perceptual similarity of different plant odours by conditioning individual carpenter ants to one odour, and subsequently testing their response to another, structurally different odour. We found that asymmetry in generalisation, where ants generalise from odour A to B, but not from B to A, is dependent on both chain length and functional group. By conditioning ants to a binary mixture, and testing their reaction to the individual components of the mixture, we show that overshadowing, where parts of a mixture are learned better than others, is rare. Additionally, generalisation is dependent not only on the structural similarity of odorants, but also on their functional value, which might play a crucial role. Our results provide insight into how ants make sense of the complex chemical world around them, for example in a foraging context, and provide a basis with which to investigate the neural mechanisms behind perceptual similarity.

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INTRODUCTION

Associative learning is widespread in the animal kingdom (Giurfa, 2007), as it allows individuals to make predictions about stimuli surrounding them and their potential meaning [e.g. bees (Bitterman et al., 1983); cephalopods (Cole and Adamo, 2005); ants (Guerrieri and d'Ettorre, 2010); mice (Watkins et al., 1998)]. The perception of relevant stimuli relies on complex mechanisms; animals are able to not only discriminate between different stimuli, but also generalise between stimuli, i.e. to consider different but similar stimuli as equivalent when they have similar ecological value (e.g. Ghirlanda and Enquist, 2003). For instance, volatiles emitted by flowers can vary slightly, but these variations are not necessarily indicative of differences in nectar quality.

Although insects have originally been neglected in studies addressing higher-order cognitive processes, during the last three decades honey bees have become model organisms for the study of learning and memory (de Brito-Sanchez et al., 2008; Giurfa, 2007) because they live in organised complex societies and show an amazing capacity for learning. Olfaction represents the most widespread sensory modality among animals (Wyatt, 2003), and thus associative learning in insects has been extensively studied in the olfactory modality. It has been difficult, though, to identify which characteristics of the odorant molecules result in generalisation to other odorant molecules. Generalisation is thought to depend on the degree of similarity between the stimuli along one or several

dimensions (Guerrieri et al., 2005b; Shepard, 1987). Guerrieri et al. established a putative olfactory space for the honey bee where two chemical features of aliphatic odorant molecules, carbon chain length and functional group, represent dimensions defining this olfactory space (Guerrieri et al., 2005b). Differences among carbon chain lengths, which can be objectively quantified, influenced the level of perceptual similarity shown by the bees. Different functional group, conversely, did not present any evident quantitative differences, which prevented ordering them in an objective hierarchical sequence, i.e. it was possible to give an increasing order to carbon chain lengths – C6, C7, C8, C9 – but establishing such a hierarchy for functional groups (primary alcohols, secondary alcohols, aldehydes and ketones) was basically impossible.

Learning about mixtures relies on complex information processing and has been extensively studied in the honey bee, both on a behavioural (Laloi et al., 2000; Laloi et al., 1999; Reinhard et al., 2010; Smith, 1998) and a neurophysiological level (Deisig et al., 2006; Joerges et al., 1997). Several theories have been proposed to account for mixture learning (for a review, see Harris, 2006). According to elemental theories such as the Rescorla–Wagner model (Rescorla and Wagner, 1972), mixtures are composed of elemental units, each of which can form an association during associative learning (mixture of AB is perceived as A+B). This theory has found support on a neurophysiological level (Deisig et al., 2006). Configural theories such as Pearce's configural model (Pearce, 1987)

assume that stimulus patterns have distinct configurations (mixture of AB is perceived as C), so an association can only be formed on the configuration as a whole. This model has found support for instance in humans (i.e. Le Berre et al., 2008a; Le Berre et al., 2008b). These theories are not mutually exclusive, and a combination is possible, where mixtures of two or more stimuli are represented by the individual elements (elemental theory), as well as an element unique to the mixture (configural theory): a mixture of AB will be perceived as ABC (e.g. Deisig et al., 2003).

These examples assume that saliency of odours is equal; however, this does not have to be the case. One result of unequal saliencies of mixture components is overshadowing (Pavlov, 1927). When learning a binary mixture, one of the components can be more salient than the other, and as a result an individual conditioned to this mixture will only learn the more salient component. For example, if A is very salient, a mixture of AB could be perceived as A alone, and thus component A overshadows component B (Kay et al., 2005; Linster and Smith, 1997; Smith, 1996). Recently, a study by Reinhard et al. showed that honey bees that are trained to associate complex mixtures of 14 compounds with sucrose reward only learn certain components of the mixture (so-called 'key odorants') (Reinhard et al., 2010). The 'key odorant hypothesis' encompasses both configural and elemental learning. While elements of the mixture are learned, none of them evokes such a strong reaction as the mixture itself, suggesting that the mixture of odorants in itself might be perceived as a configural stimulus.

Although very useful as a model organism, the honey bee by itself cannot represent the multifaceted world of insect societies, which is characterised by a multitude of life histories allowing their ecological success in a variety of ecosystems. Ants are all eusocial and have evolved what can be considered among the most advanced and complex societies. Despite this, only recently have the first steps been made in our understanding about how ants perceive and learn odours (e.g. Bos et al., 2012; Bos et al., 2010; Dupuy et al., 2006; Guerrieri et al., 2011; Guerrieri et al., 2009; Josens et al., 2009), and mixture learning has remained unexplored. We recently demonstrated that *Camponotus aethiops* ants show asymmetry in generalisation (Bos et al., 2012). Ants were successfully trained to associate a long-chain hydrocarbon to sucrose reward. In subsequent choice tests, these ants generalised to a novel hydrocarbon only when the chain length of the novel hydrocarbon was shorter than that of the conditioned one, while they discriminated between the two molecules when the test hydrocarbon had a longer carbon chain than the hydrocarbon previously associated with sucrose. Similarity among stimuli was therefore not only dependent on their structure, but also on the animal's training experience. Comparable asymmetric responses had been previously observed in honey bees (Guerrieri et al., 2005a; Guerrieri et al., 2005b; Sandoz et al., 2001).

We concluded that asymmetry in generalisation might follow an inclusion criterion [which was previously suggested in a nestmate recognition context (Guerrieri et al., 2009)], predicting that generalisation occurs when the novel stimulus is, during detection or perception, 'included' in the conditioned stimulus; e.g. when the only difference between two substances is chain length, a shorter substance is treated as a longer substance, but not the other way around (Bos et al., 2012). This could be the result of several processes, at different levels of the olfactory system. First, the binding pockets of odorant binding proteins (OBPs) might bind the molecule to which they are tuned, but also similar but shorter chain molecules as they fit into the binding pocket, while longer chain molecules do not fit. In this scenario, the conditioned stimulus and stimuli that are shorter than the conditioned stimulus will produce

a signal, while longer molecules will not elicit a response. The inclusion criterion might work also at the level of the antennal lobe. Here, the glomerular activation pattern of longer molecules could 'include' the activation pattern of shorter molecules.

We therefore wanted to investigate whether, besides chain length, the functional groups of molecules can also be assigned to a hierarchy, by using an array of volatile substances that are commercially available. Here, we test olfactory learning, generalisation and overshadowing, using the recently developed appetitive conditioning paradigm for ants, the maxilla-labium extension response (MaLER) (Guerrieri and d'Ettorre, 2010). In order to establish whether ants follow a certain 'set of rules' for generalisation of volatile substances, and whether these rules depend on chain length and functional group of the molecules encountered, we used six odorants varying in two chemical features, chain length and functional group. Odorants belonged to three chemical classes: primary alcohols, aldehydes and secondary ketones, and differed also in chain length, which was either six or eight carbon atoms long. These odours can be released by plants and thus they are biologically relevant for carpenter ants that feed on nectar (mostly from extra-floral nectaries) and would benefit from establishing an association between rewarding plants and their odour. We measured perceptual similarity of the odours by conditioning individual ants to one odour, and testing their reaction to another. We predicted that secondary ketones and aldehydes should be perceived as more similar to each other than to primary alcohols with regards to generalisation, as the functional group of both ketones and aldehydes consists of an oxygen atom connected to the carbon chain with a double bond. Overshadowing was investigated by conditioning individuals to a binary mixture, and then testing their reaction to the individual components of the mixture.

MATERIALS AND METHODS

Study organism

Six queenright colonies of *Camponotus aethiops* (Latr.), a monogynous species with workers of different sizes (minor, medium and major) were collected in spring 2009 in the Italian Apennines, near Bologna. Each colony was housed in a Fluon-coated plastic box (27×17×9.5 cm) containing a plaster floor serving as the nest area. This box was connected to another box of the same size, serving as the foraging area. The ants were fed twice a week with diluted honey and mealworms (*Tenebrio molitor*); water was provided *ad libitum*. The nests were kept in a climate-controlled room at 25±2°C under a 12 h:12 h light:dark photoperiod. Two weeks before the start of the experiment, the colonies were deprived of honey, while still receiving water and mealworms, in order to increase the ants' motivation for sugar reward during conditioning.

Experimental setup

Preparation

Medium-sized worker ants were taken from the foraging arena and anaesthetised on ice for 10 min before they were harnessed individually in an ant holder. This holder was an inverted 0.2 ml Eppendorf microcentrifuge tube, from which the tip was cut off to create a second, smaller opening. The ant's head was passed through this opening in the tube, and fixed with adhesive tape. In this way ants could only move their antennae and mouthparts (see Guerrieri and d'Ettorre, 2008). The harnessed ants were left undisturbed for approximately 3 h to habituate to the experimental conditions. Afterwards, their motivation for sucrose was checked by presenting each individual with a droplet of sucrose solution (50% w/w), and

only individuals responding to this presentation with MaLER were used for the experiments.

Stimuli

The unconditioned stimulus (US) used was sucrose solution (50% w/w). Conditioned stimuli (CS) were six different odourants – 1-octanol, 1-hexanol, octanal, hexanal, 2-octanone and 2-hexanone (Sigma-Aldrich, Broendby, Denmark) – differing in chain length, functional group or both (Table 1). Stimuli were standardised according to volatility by diluting them in mineral oil (supplementary material Table S1), so that each stimulus released the same amount of molecules into the air, in order to prevent concentration differences during conditioning. Before each training phase, 5 µl of CS were applied onto individual clean strips of filter paper (1 cm²) and placed inside individual 10 ml plastic syringes, applying a standard procedure previously used for conditioning ants (Guerrieri and d’Ettorre, 2010) and honey bees (Guerrieri et al., 2005b; Vergoz et al., 2007). For single substance learning, the CS consisted of a single stimulus. For mixture learning, a binary mixture was created by applying 5 µl of two stimuli onto a single strip of filter paper; every possible binary mixture using the six substances was tested.

Conditioning trials

Absolute conditioning was achieved by training individual ants to the CS for four consecutive trials. Each conditioning trial lasted 1 min. Fifteen seconds after being placed under a stereomicroscope, the ant received the CS for 5 s. This was presented by placing the syringe 2 cm from the head of the ant, and gently blowing 10 ml of air towards the antennae. Presentation of the US started 3 s after the onset of the CS, and lasted for 5 s, thus creating a 2 s overlap between the CS and the US. Ten individuals were tested in series; therefore, the inter-trial interval was 10 min. An air extractor was placed behind the ant, in order to remove the volatile substances from the experimental area. Learning was scored by observing extension of the maxilla-labium, resulting in a binary response variable (yes/no).

Test

For single substance learning, the test consisted of four randomised test trials: presentation of the CS, a novel stimulus (test stimulus, TS), a mixture of the CS and the TS, and a control (clean air). For mixture learning, the test consisted of the following four randomised test trials: presentation of the CS (a binary mixture), presentation of each of the two individual substances contained in the mixture, and presentation of clean air. After the test, each individual was presented with a droplet of sucrose solution. Only those individuals that still responded to this stimulation were included in the statistical analysis of the test (98%). In total, 611 ants were successfully conditioned to a single substance, and 329 ants were conditioned to a binary mixture. For both single substance learning and mixture

learning, the test took place 10 min after conditioning, allowing us to interpret our results in light of short-term memory.

Statistical analysis

Conditioning trials

We quantified the conditioned response as MaLER to the given stimulus, resulting in a binary score depicting success or failure to respond to the stimulus. Learning performances were analysed using a generalised linear mixed model (GLMM) with binomial error structure and logit link function. In this model, we included trial number and CS, as well as the interaction between them as fixed factors, and individual as a random effect. The effect of trial number might, to some extent, be individually specific, and therefore we included a random effects structure allowing for random intercepts, random slopes, as well as a correlation between them. This model was run separately for single substance learning and mixture learning. Significance of interaction terms was determined by comparing the full model with a reduced model excluding the interaction, using a likelihood ratio test (LRT). To test whether, during the test, all conditioned stimuli had been learned at a similar level we used a generalised linear model (GLM) with binomial error structure and logit link function, including the CS as a fixed effect.

Test

Single substance learning

Of the 611 ants conditioned to a single pure substance, 491 individuals (80%) responded to the CS in the test, and were thus included in the subsequent analysis. For testing the reaction to single substances after single substance learning, we used a GLM with a binomial error structure and logit link function. In this model we included functional group (whether the functional group of the CS and TS was similar) and chain length (whether the chain length of the CS was longer, shorter or equal to the TS) as fixed factors, as well as the interaction between them. This allowed us to investigate the effect of functional group and chain length, and test whether an inclusion criterion exists, and whether this inclusion criterion is only dependent on chain length, or also on functional group. We checked stability of each model by excluding data points one by one from the data and comparing the estimates derived with those obtained for the full model, which indicated that no datapoints that significantly influence the stability of the model exist. All models were fitted in R (R Development Core Team, 2011) using the function lmer of the R-package lme4 (Maechler and Bates, 2010).

Mixture learning

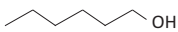
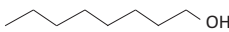
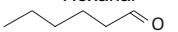

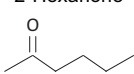
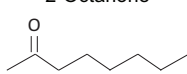
Of the 329 ants conditioned to a binary mixture, 284 individuals (86%) responded to the CS in the test, and were thus included in the subsequent analysis. For testing whether one of the components of a binary mixture is learned better than the other one, or whether both components are equally learned, we used Fisher’s exact tests for each of the mixtures.

RESULTS

Conditioning trials

For single substance conditioning, we found a significant interaction between trial number and response to the CS [likelihood ratio test (LRT), $\chi^2=18.87$, d.f.=5, $P<0.01$; Fig. 1], suggesting that some substances (e.g. octanol) are learned better than others (e.g. 2-octanone). During the test phase, there was a non-significant trend to respond differently to each of the substances (LRT, $\chi^2=10.04$, d.f.=5, $P=0.07$), again suggesting the possibility that some substances are learned better than others.

Table 1. Volatile substances used in this study, grouped by functional group and chain length

	Six-carbon chain	Eight-carbon chain
Primary alcohol	1-Hexanol 	1-Octanol 
Aldehyde	Hexanal 	Octanal 
Secondary ketone	2-Hexanone 	2-Octanone 

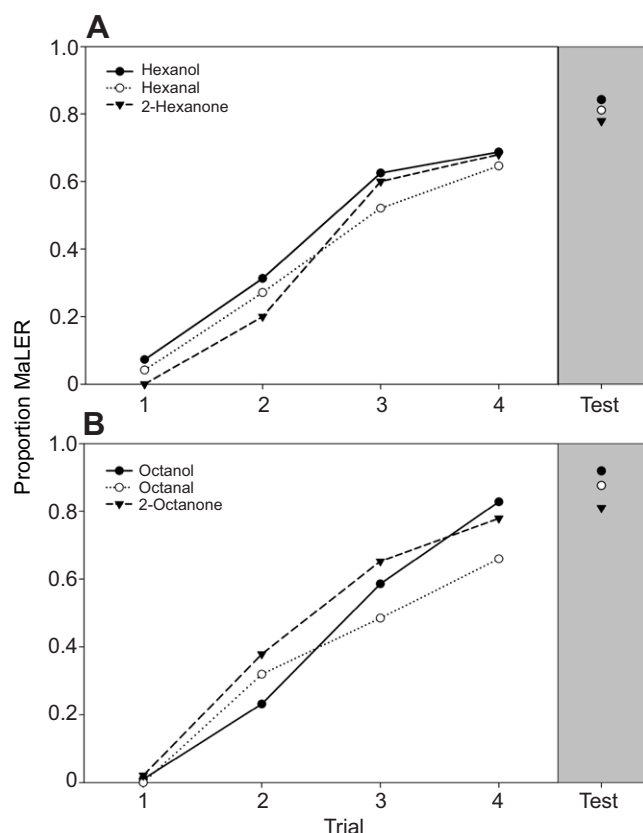


Fig. 1. Conditioning to single substances. Acquisition curves of single substances in the course of four conditioning trials, as well as the average response during the test for conditioned stimuli with a six-carbon chain (A) and an eight-carbon chain (B). A significant interaction between trial number and response to conditioned stimulus (LRT, $\chi^2=18.87$, d.f.=5, $P<0.01$) was found, suggesting that some substances (e.g. octanol) are learned better than others (e.g. 2-octanone). During the test phase, there was a non-significant trend to respond differently to each of the substances (LRT, $\chi^2=10.04$, d.f.=5, $P=0.07$), again suggesting the possibility that some substances are learned better than others.

The acquisition of binary mixtures was not significantly dependent on the composition of the mixture (LRT, $\chi^2=21.23$, d.f.=14, $P=0.1$; Fig. 2), but it depended only on trial number (GLMM, $z=16.46$, $P<0.001$). During the test phase, however, there was a significant effect of CS on the response (LRT, $\chi^2=32.25$, d.f.=14, $P<0.01$). This suggests that the composition of the mixture affects learning efficiency, even if acquisition curves do not differ statistically.

Test

Single substance learning

Only ants that reacted to the CS in the test were included in the analysis. The reaction of ants to a single TS after single substance conditioning showed a significant interaction between chain length and functional group (LRT, $\chi^2=4.66$, d.f.=1, $P=0.03$; Fig. 3), showing that both chain length and functional group influence generalisation between stimuli.

However, for the reaction to a binary mixture, comprising the CS and a novel TS, no such interaction was found (LRT, $\chi^2=2.26$, d.f.=1, $P=0.13$), and it was thus removed from the model. Only the effect of chain length was significant (LRT, $\chi^2=111.150$, d.f.=2, $P<0.001$), while the effect of functional group was not (GLM,

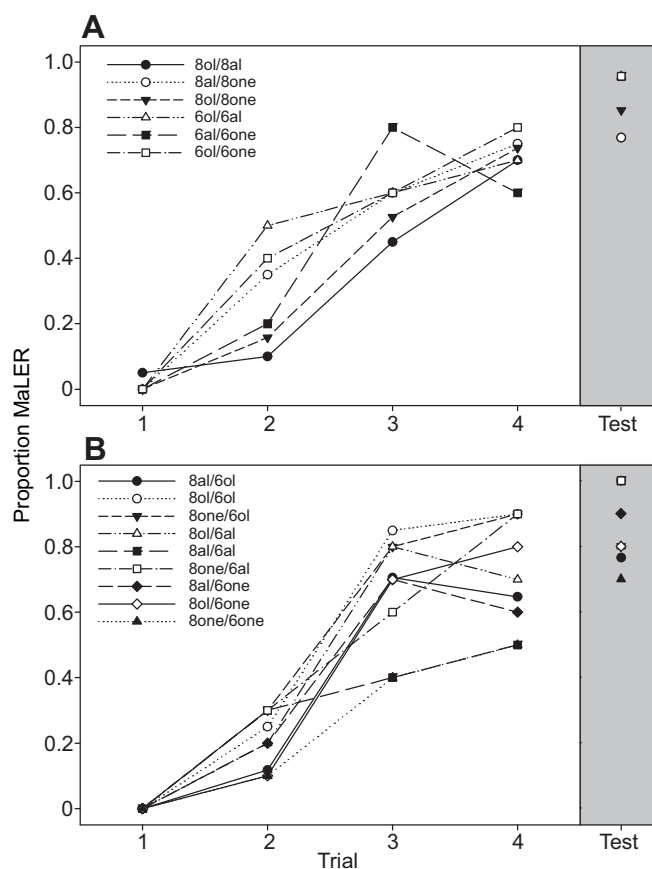


Fig. 2. Conditioning to binary mixtures. Acquisition curves of binary mixtures in the course of four conditioning trials, as well as the average response during the test. Acquisition was not significantly dependent on the composition of the mixture (LRT, $\chi^2=21.23$, d.f.=14, $P=0.1$), but depended only on trial number (GLMM, $z=16.46$, $P<0.001$). During the test phase, there was a significant effect of conditioned stimulus on the response (LRT, $\chi^2=32.25$, d.f.=14, $P<0.01$), suggesting that the composition of the mixture affects learning efficiency, even if acquisition curves do not differ statistically. (A) Acquisition curves of mixtures containing either six-chain or eight-chain molecules. (B) Acquisition curves of mixtures containing a six-chain and an eight-chain molecule.

$z=1.21$, $P=0.23$). In Fig. 4, we can see for instance that when a substance with an eight-carbon chain is learned (1-octanol, octanal or 2-octanone), generalisation to a mixture including substances with a six-carbon chain occurs (but not the other way around), illustrating the importance of chain length in generalisation.

Mixture learning

For three out of 15 conditioned binary mixtures, ants did not respond equally to the two components in the test phase, when presented individually. These mixtures were: 1-octanol/2-hexanone, 1-hexanol/hexanal and 1-hexanol/2-hexanone (Fisher's exact test, two tailed, $P<0.01$ in all three cases; Fig. 5). For all the other binary mixtures, no significant effect was found (Fisher's exact test, two tailed, $P=0.21$ –1.00), suggesting that in these cases overshadowing does not occur.

DISCUSSION

By using only four training trials, individual ants could be conditioned to all substances used in this study, independently of chain length and functional group. After conditioning, approximately

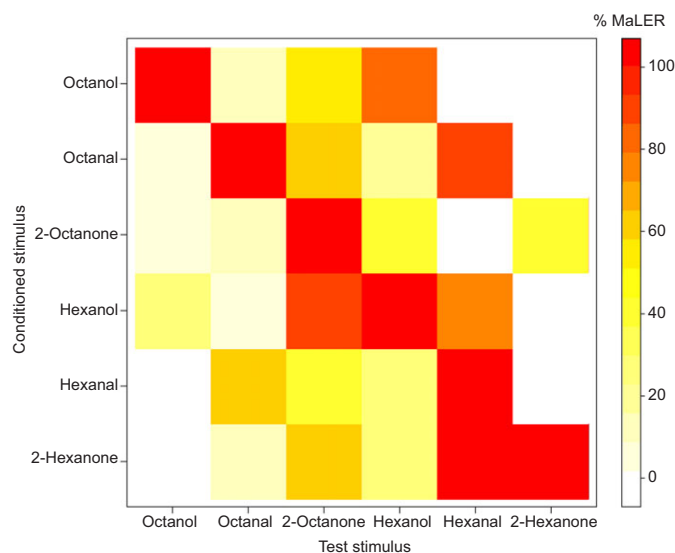


Fig. 3. Generalisation matrix: single substance conditioning, single substance test. Generalisation towards novel substances, after conditioning to a single substance. The darker the colour of the square, the greater the percentage of individuals that responded with the maxilla-labium extension response (MaLER). There is a significant interaction between chain length and functional group (LRT, $\chi^2=4.66$, d.f.=1, $P=0.03$), indicating that both chain length and functional group influence generalisation between stimuli.

80% of the trained individuals responded to the CS in a non-reinforced test. In the case of single substance learning, the significant interaction between trial number and CS suggests that the efficacy of learning is dependent on the CS. Indeed, acquisition was faster when 2-octanone or 1-octanol was the CS. Despite the

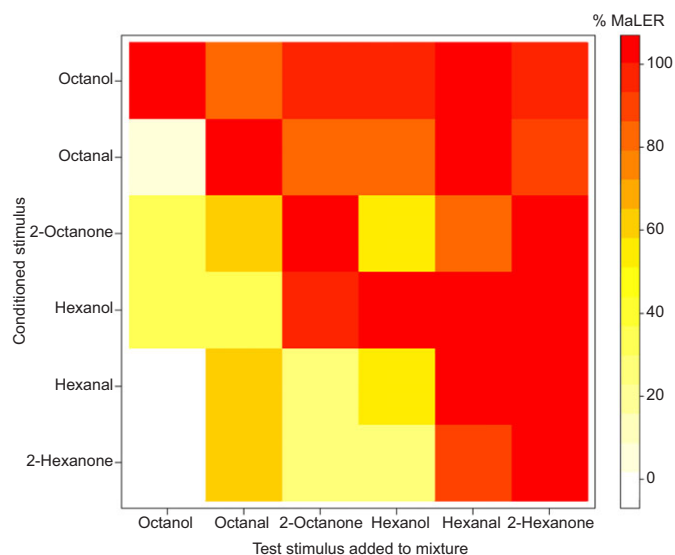


Fig. 4. Generalisation matrix: single substance conditioning, binary mixture test. Generalisation towards a mixture containing the conditioned stimulus and a novel stimulus. The darker the colour of the square, the greater the percentage of individuals that responded with MaLER. Only the effect of chain length was significant (LRT, $\chi^2=111.150$, d.f.=2, $P<0.001$), while the effect of functional group was not (GLM, $z=1.21$, $P=0.23$). For instance, when a substance with an eight-carbon chain is learned (octanol, octanal or 2-octanone), generalisation to a mixture including substances with a six-carbon chain occurs (but not the other way around), illustrating the importance of chain length in generalisation.

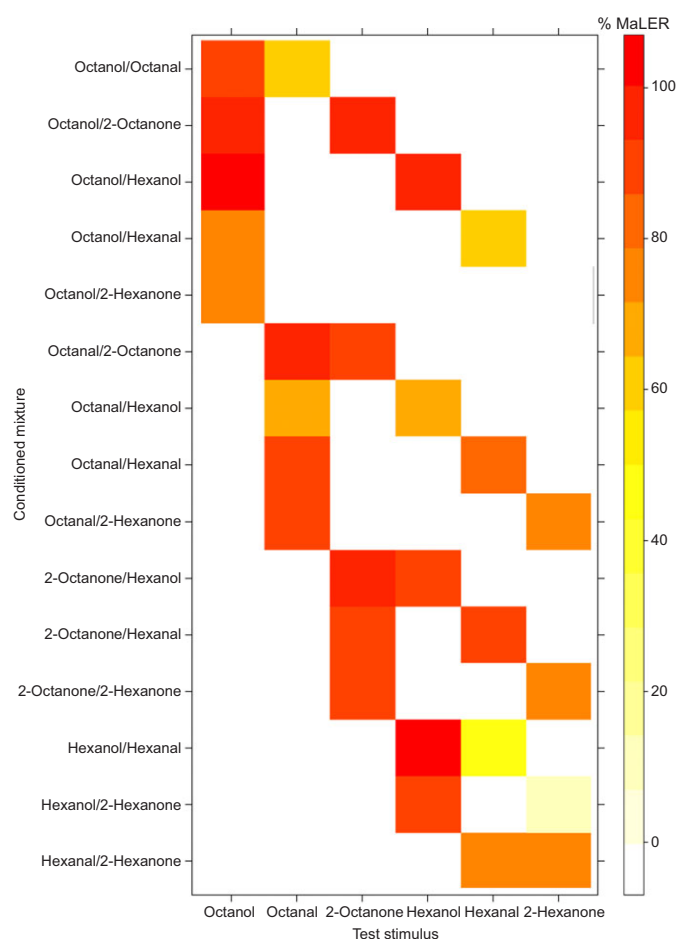


Fig. 5. Generalisation matrix: binary mixture conditioning, single substance test. Generalisation towards the single components of a binary mixture, after conditioning to a binary mixture. The darker the colour of the square, the greater the percentage of individuals that responded with MaLER. Overshadowing only occurs in three out of 15 binary mixtures (octanol/2-hexanone, hexanol/hexanal and hexanol/2-hexanone) (Fisher's exact test, two tailed, $P<0.01$ in all cases).

fact that some substances are learned more efficiently than others, during the subsequent test the response to all substances was comparable. Thus, the rate of acquisition did not significantly influence the response during the test, suggesting that a plateau (maximum level of learning) can be reached with four training trials. However, when the CS was not a single substance, but a binary mixture, significant differences in response during the test were found, suggesting that some mixtures are learned better than others.

The response to single substances after absolute conditioning to a single substance yielded a significant interaction between chain length and functional group, indicating that the effect of functional group is dependent on whether the chain length is shorter or longer. Indeed, generalisation (i.e. treating different stimuli as similar) mostly occurs when not only the functional group is similar, but also when the chain length of the TS is shorter than that of the CS. This phenomenon, where generalisation does not occur equally between substances, but depends on the animal's learning experience, is named asymmetric generalisation. Asymmetry in generalisation has previously been described in honey bees for volatile substances (Guerrieri et al., 2005a; Guerrieri et al., 2005b; Sandoz et al., 2001) and in ants for long-chain hydrocarbons (Bos et al., 2012). Asymmetrical generalisation can be explained by the inclusion criterion model (Bos et al., 2012; Guerrieri

et al., 2009), where generalisation occurs when the novel stimulus is, during detection or perception, 'included' in the CS, e.g. a shorter substance is treated as a longer substance of the same kind. In a previous work, we found that two long-chain hydrocarbons are generalised only when the chain length of the TS is shorter than that of the CS (Bos et al., 2012). The present study provides an extension of the inclusion criterion because we show not only that the chain length of the TS has to be shorter than that of the CS, but also that the functional group of the two substances has to be similar for generalisation to occur.

A special case appears to be 2-octanone: no matter what the CS was, generalisation towards 2-octanone occurred in every case, which suggests that this odorant might have some inherent functional value for the ants. Indeed, 2-octanone has been found to be a component of the alarm pheromone in some species of *Atta* (Hughes et al., 2001; Moser et al., 1968), and 3-octanone, but not 2-octanone, has been described as a component of the alarm pheromone of *Camponotus schaefferi* (Duffield and Blum, 1975); whether this is also the case for *C. aethiops* remains to be investigated. A recent study found a peculiar effect of 2-octanone in *Camponotus fellah* ants that were differentially conditioned to chemical substances in a Y-maze. After conditioning, one would expect a preference for the CS+ over the CS-, and preference for the CS+ over a novel stimulus, while the novel stimulus would be preferred over the CS-. When 2-octanone was the novel stimulus, however, the CS- was preferred (Josens et al., 2009). In our case, instead of showing an inhibitory effect, generalisation to 2-octanone was more frequent than expected. This implies that similarity in the neural representation of two odorants is not purely based on their chemical similarity, but might also depend on their functional value (Guerrieri et al., 2005a; Sandoz et al., 2001). If 2-octanone were a pheromone component for *C. aethiops*, detected by a specific pheromone receptor protein, which would be narrowly tuned, no generalisation towards other odours would be expected when 2-octanone was presented as the CS, and this is indeed what we find. However, when 2-octanone was presented as the TS, generalisation occurred regardless of the CS. This generalisation would not be expected, and we therefore assume that 2-octanone is likely not detected by a specific pheromone receptor protein in *C. aethiops*.

When ants were conditioned to single substances and tested with binary mixtures, comprising the CS and a novel TS, only the effect of chain length was significant, while the effect of functional group was not. Indeed, generalisation towards the mixture only occurs when the novel stimulus in the mixture has an equal or shorter chain length than the CS. However, when the chain length of the two substances in the mixture is equal, a difference in saliency between functional groups can appear. Generalisation mostly occurs when a primary alcohol (1-octanol, 1-hexanol) is the CS. When instead an aldehyde (octanal, hexanal) is the CS, presentation of a mixture of the CS and a primary alcohol inhibits the response. When a secondary ketone (2-octanone, 2-hexanone) is the CS, presentation of a mixture of the CS and either a primary alcohol or aldehyde will inhibit the response. These results allow us to establish a hierarchy, where alcohols are the most salient substances, followed by aldehydes, while secondary ketones are the least salient. These primary alcohols, besides being plant odours, may also occur as components of the alarm pheromone in some ants [e.g. *Oecophylla longinoda* (Bradshaw et al., 1975)] and in the honey bee (e.g. Kraus, 1990; Wager and Breed, 2000), which could explain their relevance for our ants.

The fact that secondary ketones are treated as more similar to aldehydes than alcohols is possibly a result of the similarity in

functional group, as both aldehydes and secondary ketones contain a similar functional group, which only differs by its position on the carbon chain. Similar functional groups will result in aldehydes and secondary ketones having a similar stereochemistry, allowing both kinds of molecules to fit similar odorant binding proteins, because of the adapted three-dimensional shape of their binding pocket.

When conditioned to binary mixtures, ants generally responded to each of the components presented separately in the subsequent test. This suggests that the differences in saliencies observed during the previous experiment did not produce any overshadowing effect [also found in newborn rabbits (Coureaud et al., 2009)]. The fact that for our ants each component of the conditioned binary mixture was associated with food suggests elemental learning instead of configural learning as the underlying model. In three out of the 15 cases, however, an overshadowing effect was found. These mixtures were 1-octanol/2-hexanone, 1-hexanol/hexanal and 1-hexanol/2-hexanone, all involving a primary alcohol, thus indicating that this class of substance can produce an overshadowing effect by its saliency. Nevertheless, overshadowing was only partial in two out of the three cases. Kay et al. suggested that overshadowing results from 'masking' during learning, which could be the result of intensity differences or interactions at the receptor or glomerular level between the components (Kay et al., 2005). These three cases thus have more in common with the 'key odorant hypothesis' than with elemental learning model; despite this, the key odorant model does not appear to be the general rule in *C. aethiops*, at least in the case of binary mixtures. Further studies using mixtures containing more than two components (see Reinhard et al., 2010) would allow better elucidation of the role of functional groups in overshadowing.

It is worth noting that the level of generalisation from the conditioned odour to the mixture containing the CS, and from the mixture to the single components, is always lower than the response to the CS alone, and that the amount of difference in generalisation depends on the specific combination of the odours (e.g. when 1-octanol is the CS, and the mixture of 1-octanol and octanal is the TS, the response to the TS is lower than to the CS; also, when the mixture of 1-octanol and octanal is the CS, and the individual components are tested, the response to the CS is higher than to the individual components). This suggests that there is some configural processing of mixtures, leading to mixtures having novel odour qualities, which are only partially similar to the quality of the single components (e.g. Deisig et al., 2003). This phenomenon has previously been found in rats (Linster and Smith, 1999), and implies a weak configural computation of the binary mixture (Kay et al., 2005). Alternative hypotheses remain, at present, unexplored. Task unevenness, for instance, which is the difference of conditions between the conditioning phase and test phase (single odour *versus* mixture or *vice versa*), might play a role. In the case of generalisation from a single CS to a mixture, there could also be a novelty effect, where the unfamiliar odour in a mixture containing the CS has an inhibitory effect.

In conclusion, we found a hierarchy of saliencies, where primary alcohols are the most salient, followed by aldehydes and secondary ketones, respectively. It remains to be investigated whether this is a mere side effect of the neurophysiological mechanisms underlying detection and perception by the ant, or whether it has an adaptive value. Investigating the occurrence and function of the different types of substances present in the natural chemical landscape of *C. aethiops* [e.g. plants, as it is a nectivorous species (Dupuy et al., 2006)] might reveal the underlying adaptive reason of the described stimulus hierarchy. The present study, together with our previous results (Bos et al., 2012), provides a fundamental step forward to

unravel the mechanisms and function of asymmetry in generalisation. We predict that the inclusion criterion might originate at two different levels: (1) OBPs tuned to a certain compound (i.e. 1-octanol) could be sensitive to shorter compounds (i.e. 1-hexanol), but not to longer ones, as shorter molecules can fit into binding pockets of OBPs tuned to longer molecules, but longer molecules will not fit into binding pockets of OBPs tuned to shorter molecules; and (2) glomerular activation patterns in the antennal lobe of longer compounds could 'include' the activation patterns of shorter compounds, resulting in the observed asymmetry in generalisation. Further studies should reveal whether the behavioural responses found here correlate with the reaction of antennal receptors. Also, developing opto-physiological techniques (Brandstaetter et al., 2011; Zube et al., 2008) in order to study activation patterns in the antennal lobe of ants would allow testing the second prediction.

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AUTHOR CONTRIBUTIONS

N.B., P.d'E. and F.G. conceived and designed the experiments; N.B. performed the experiments; N.B. analyzed the data; N.B., P.d'E. and F.G. wrote the paper.

COMPETING INTERESTS

No competing interests declared.

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REFERENCES

- Bitterman, M. E., Menzel, R., Fietz, A. and Schäfer, S. (1983). Classical conditioning of proboscis extension in honeybees (*Apis mellifera*). *J. Comp. Psychol.* **97**, 107-119.
- Bos, N., Guerrieri, F. J. and d'Ettorre, P. (2010). Significance of chemical recognition cues is context dependent in ants. *Anim. Behav.* **80**, 839-844.
- Bos, N., Dreier, S., Jørgensen, C. G., Nielsen, J., Guerrieri, F. J. and d'Ettorre, P. (2012). Learning and perceptual similarity among cuticular hydrocarbons in ants. *J. Insect Physiol.* **58**, 138-146.
- Bradshaw, J. W. S., Baker, R. and Howse, P. E. (1975). Multicomponent alarm pheromones of the weaver ant. *Nature* **258**, 230-231.
- Brandstaetter, A. S., Rössler, W. and Kleineidam, C. J. (2011). Friends and foes from an ant brain's point of view – neuronal correlates of colony odors in a social insect. *PLoS ONE* **6**, e21383.
- Cole, P. D. and Adamo, S. A. (2005). Cuttlefish (*Sepia officinalis*: Cephalopoda) hunting behavior and associative learning. *Anim. Cogn.* **8**, 27-30.
- Coureaud, G., Hamdani, Y., Schaal, B. and Thomas-Danguin, T. (2009). Elemental and configural processing of odour mixtures in the newborn rabbit. *J. Exp. Biol.* **212**, 2525-2531.
- de Brito-Sanchez, M. G., Deisig, N., Sandoz, J. C. and Giurfa, M. (2008). Neurobiology of olfactory communication in a social insect: the honeybee. In *Sociobiology of Communication: an Interdisciplinary Perspective* (ed. P. d'Ettorre and D. P. Hughes), pp. 119-138. Oxford: Oxford University Press.
- Deisig, N., Lachnit, H., Sandoz, J. C., Lober, K. and Giurfa, M. (2003). A modified version of the unique cue theory accounts for olfactory compound processing in honeybees. *Learn. Mem.* **10**, 199-208.
- Deisig, N., Giurfa, M., Lachnit, H. and Sandoz, J. C. (2006). Neural representation of olfactory mixtures in the honeybee antennal lobe. *Eur. J. Neurosci.* **24**, 1161-1174.
- Duffield, R. M. and Blum, M. S. (1975). Identification, role and systematic significance of 3-octanone in the carpenter ant, *Camponotus schaefferi* Wm. *Comp. Biochem. Physiol.* **51B**, 281-282.
- Dupuy, F., Sandoz, J. C., Giurfa, M. and Josens, R. (2006). Individual olfactory learning in *Camponotus* ants. *Anim. Behav.* **72**, 1081-1091.
- Ghirlanda, S. and Enquist, M. (2003). A century of generalization. *Anim. Behav.* **66**, 15-36.
- Giurfa, M. (2007). Behavioral and neural analysis of associative learning in the honeybee: a taste from the magic well. *J. Comp. Physiol. A* **193**, 801-824.
- Guerrieri, F. J. and d'Ettorre, P. (2008). The mandible opening response: quantifying aggression elicited by chemical cues in ants. *J. Exp. Biol.* **211**, 1109-1113.
- Guerrieri, F. J. and d'Ettorre, P. (2010). Associative learning in ants: conditioning of the maxilla-labium extension response in *Camponotus aethiops*. *J. Insect Physiol.* **56**, 88-92.
- Guerrieri, F. J., Lachnit, H., Gerber, B. and Giurfa, M. (2005a). Olfactory blocking and odorant similarity in the honeybee. *Learn. Mem.* **12**, 86-95.
- Guerrieri, F. J., Schubert, M., Sandoz, J. C. and Giurfa, M. (2005b). Perceptual and neural olfactory similarity in honeybees. *PLoS Biol.* **3**, e60.
- Guerrieri, F. J., Nehring, V., Jørgensen, C. G., Nielsen, J., Galizia, C. G. and d'Ettorre, P. (2009). Ants recognize foes and not friends. *Proc. Biol. Sci.* **276**, 2461-2468.
- Guerrieri, F. J., d'Ettorre, P., Devaud, J.-M. and Giurfa, M. (2011). Long-term olfactory memories are stabilised via protein synthesis in *Camponotus fellah* ants. *J. Exp. Biol.* **214**, 3300-3304.
- Harris, J. A. (2006). Elemental representations of stimuli in associative learning. *Psychol. Rev.* **113**, 584-605.
- Hughes, W. O. H., Howse, P. E. and Goulson, D. (2001). Mandibular gland chemistry of grass-cutting ants: species, caste, and colony variation. *J. Chem. Ecol.* **27**, 109-124.
- Joerges, J., Kuettner, A., Galizia, G. and Menzel, R. (1997). Representations of odours and odour mixtures visualized in the honeybee brain. *Nature* **387**, 285-288.
- Josens, R., Eschbach, C. and Giurfa, M. (2009). Differential conditioning and long-term olfactory memory in individual *Camponotus fellah* ants. *J. Exp. Biol.* **212**, 1904-1911.
- Kay, L. M., Crk, T. and Thorngate, J. (2005). A redefinition of odor mixture quality. *Behav. Neurosci.* **119**, 726-733.
- Kraus, B. (1990). Effects of honey-bee alarm pheromone compounds on the behaviour of *Varroa jacobsoni*. *Apidologie (Celle)* **21**, 127-134.
- Laloi, D., Roger, B., Blight, M. M., Wadhams, L. J. and Pham-Delegue, M.-H. (1999). Individual learning ability and complex odor recognition in the honey bee, *Apis mellifera* L. *J. Insect Behav.* **12**, 585-597.
- Laloi, D., Bailez, O., Blight, M., Roger, B., Pham-Delegue, M.-H. and Wadhams, L. J. (2000). Recognition of complex odors by restrained and free-flying honeybees, *Apis mellifera*. *J. Chem. Ecol.* **26**, 2307-2319.
- Le Berre, E., Béno, N., Ishii, A., Chabanet, C., Etiévant, P. and Thomas-Danguin, T. (2008a). Just noticeable differences in component concentrations modify the odor quality of a blending mixture. *Chem. Senses* **33**, 389-395.
- Le Berre, E., Thomas-Danguin, T., Béno, N., Coureaud, G., Etiévant, P. and Prescott, J. (2008b). Perceptual processing strategy and exposure influence the perception of odor mixtures. *Chem. Senses* **33**, 193-199.
- Linster, C. and Smith, B. H. (1997). A computational model of the response of honey bee antennal lobe circuitry to odor mixtures: overshadowing, blocking and unblocking can arise from lateral inhibition. *Behav. Brain Res.* **87**, 1-14.
- Linster, C. and Smith, B. H. (1999). Generalization between binary odor mixtures and their components in the rat. *Physiol. Behav.* **66**, 701-707.
- Maechler, M. and Bates, D. (2010). *lme4: Linear Mixed-effects Models Using Eigen and R* package version 0.99937-35. <http://cran.r-project.org/web/packages/lme4/index.html>
- Moser, J. C., Brownlee, R. C. and Silverstein, R. (1968). Alarm pheromones of the ant *Atta texana*. *J. Insect Physiol.* **14**, 529-535.
- Pavlov, I. P. (1927). *Conditioned Reflexes*. Oxford: Oxford University Press.
- Pearce, J. M. (1987). A model for stimulus generalization in Pavlovian conditioning. *Psychol. Rev.* **94**, 61-73.
- R Development Core Team (2011). *R: A Language and Environment for Statistical Computing*. Vienna, Austria: R Foundation for Statistical Computing.
- Reinhard, J., Sinclair, M., Srinivasan, M. V. and Claudianos, C. (2010). Honeybees learn odour mixtures via a selection of key odorants. *PLoS ONE* **5**, e9110.
- Rescorla, R. A. and Wagner, A. R. (1972). A theory of Pavlovian conditioning: variations in the effectiveness of reinforcement and nonreinforcement. In *Classical Conditioning II* (ed. A. H. Black and W. F. Prokasy), pp. 64-99. New York, NY: Appleton-Century-Crofts.
- Sandoz, J. C., Pham-Delegue, M.-H., Renou, M. and Wadhams, L. J. (2001). Asymmetrical generalisation between pheromonal and floral odours in appetitive olfactory conditioning of the honey bee (*Apis mellifera* L.). *J. Comp. Physiol. A* **187**, 559-568.
- Shepard, R. N. (1987). Toward a universal law of generalization for psychological science. *Science* **237**, 1317-1323.
- Smith, B. H. (1996). The role of attention in learning about odorants. *Biol. Bull.* **191**, 76-83.
- Smith, B. H. (1998). Analysis of interaction in binary odorant mixtures. *Physiol. Behav.* **65**, 397-407.
- Vergoz, V., Roussel, E., Sandoz, J. C. and Giurfa, M. (2007). Aversive learning in honeybees revealed by the olfactory conditioning of the sting extension reflex. *PLoS ONE* **2**, e288.
- Wager, B. R. and Breed, M. D. (2000). Does honey bee sting alarm pheromone give orientation information to defensive bees? *Ann. Entomol. Soc. Am.* **93**, 1329-1332.
- Watkins, R. W., Gurney, J. E. and Cowan, D. P. (1998). Taste-aversion conditioning of house mice (*Mus domesticus*) using the non-lethal repellent, cinnamamide. *Appl. Anim. Behav. Sci.* **57**, 171-177.
- Wyatt, T. D. (2003). *Pheromones and Animal Behaviour: Communication by Smell and Taste*. Cambridge: Cambridge University Press.
- Zube, C., Kleineidam, C. J., Kirschner, S., Neef, J. and Rössler, W. (2008). Organization of the olfactory pathway and odor processing in the antennal lobe of the ant *Camponotus floridanus*. *J. Comp. Neurol.* **506**, 425-441.

Table S1. The amount of paraffin oil and substance (in μl) used to standardize the stimuli according to their volatility; this ensured all stimuli had the same vapour pressure during the experiment

Substance	Vapour pressure (mmHg)	1/Vapour pressure	Substance (μl)	Paraffin oil (μl)
1-Hexanol	0.928	1.078	10.776	989.224
1-Octanol	0.0794	12.594	125.945	874.055
Octanal	1.18	0.847	8.475	991.525
2-Octanone	1.35	0.741	7.407	992.593
Hexanal	11.3	0.088	0.885	999.115
2-Hexanone	11.6	0.086	0.862	999.138