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Olfactory Discrimination Ability and Odor Structure–Activity Relationships in Honeybees

Matthias Laska, C. Giovanni Galizia[1], Martin Giurfa[1] and Randolf Menzel[1]

Institut für Medizinische Psychologie, Ludwig-Maximilians–Universität München, Goethestraße 31, D-80336 München and [1]Institut für Neurobiologie, Freie Universität Berlin, Königin-Luise-Straße 28–30, D-14195 Berlin, Germany

Correspondence to be sent to: Matthias Laska, Institut für Medizinische Psychologie, Ludwig-Maximilians–Universität, Goethestraße 31, D-80336 München, Germany. e-mail: Laska@imp.med.uni-muenchen.de

Abstract

Using the training procedure introduced by von Frisch in 1919, we tested the ability of free-flying honeybees to discriminate a conditioning odor from an array of 44 simultaneously presented substances. The stimuli included homologous series of aliphatic alcohols, aldehydes and ketones, isomeric forms of some of these substances, as well as several terpenes and odor mixtures, and thus comprised stimuli of varying degrees of structural similarity to any conditioning odor. We found (i) that the honeybees significantly distinguished between 97.0% of the 1848 odor pairs tested, thus showing an excellent discrimination performance when tested in a free-flying situation with an array of structurally related substances; (ii) a significant negative correlation between discrimination performance and structural similarity of odorants in terms of differences in carbon chain length with all aliphatic substance classes tested; (iii) that both the position and type of a functional group also affected discriminability of odorants in a substance class-specific manner; and (iv) striking similarities in odor structure–activity relationships between honeybees and human and nonhuman primates tested previously on a subset of substances employed here. Our findings demonstrate that the similiarities found in the structural organization of the olfactory systems of insects and vertebrates are paralleled by striking similarities in relative discrimination abilities. This strongly suggests that similar mechanisms of odor coding and discrimination may underlie olfaction in vertebrates and insects.

Introduction

Animals of most species are capable of discriminating between a variety of odors. This ability is often crucial for the organization of feeding, mating and social communication, as well as for the processes of learning and memory that are associated with these behaviors. Thus, in order to understand these behaviors and processes, it is important to gain insight into the neural mechanisms underlying the discrimination of odors. Several lines of evidence suggest that odor discrimination begins with differential interaction of odor molecules with different types of olfactory receptors (Buck and Axel, 1991; Hildebrand and Shepherd, 1997). However, there is still only sparse information as to which properties of an odor molecule are functional in determining the degree of interaction with a given receptor, and thus in determining its perceived odor quality (Ohloff *et al.*, 1991).

In a series of psychophysical studies aimed at assessing the ability to discriminate between members of homologous series of aliphatic substances, one of us has recently shown that significant correlations between structural similarities in terms of differences in carbon chain length and odor quality exist both in human and in nonhuman primates (Laska and Freyer, 1997; Laska and Teubner, 1998, 1999b) (M. Laska *et al.*, submitted for publication). It is clear, however, that carbon chain length is only one of presumably several determinants of the interaction between odor stimuli and olfactory receptors, and that other molecular properties, such as position or type of functional groups, may also affect odor quality and thus discriminability.

Considering the striking similarities in the structural organization of the olfactory systems in vertebrates and insects (Boeckh *et al.*, 1990), it seems reasonable to assume that similar principles of odor coding and discrimination apply to both groups of animals. In order to test this hypothesis we sought to assess olfactory discrimination ability in honeybees, one of the model organisms for the study of insect olfaction and olfactory learning (Menzel and Müller, 1996), for a set of substances of varying structural similarity.

Olfactory performance in honeybees was first investigated by von Frisch (von Frisch, 1919). He simultaneously offered several odorants to free-flying honeybees, of which one was associated with a sugar solution as a reward. The bees were strongly attracted by the substance associated with sugar, and this conditioning remained after the suppression of the

reward, even though the respective positions of the different odors were changed. von Frisch found that the animals clearly discriminated 28 out of 32 pairs of odors and partially confused the four others, for which some similarity of odor quality was observed in man. Thus, he concluded that odor perception in bees might be similar to that of man.

Although numerous studies using different approaches from the behavioral to the molecular level have investigated various aspects of honeybee olfaction following von Frisch's pioneering work, and at least some studies have assessed olfactory discrimination performance in this species (Vareschi, 1971; Pham-Delegue *et al.*, 1993), only a few have so far directly addressed odor structure–activity relationships in honeybees (Smith and Menzel, 1989; Sachse *et al.*, 1998). However, new findings on olfactory coding at the level of the antennal lobes (an equivalent of the vertebrate olfactory bulb) have shown that different odors are represented in terms of different glomerular activation patterns (Joerges *et al.*, 1997). These findings make it worthwhile to compare the behavioral performance with the neurobiological one, to analyse to what extent the neural coding is translated into a behavioral response.

We decided to take up von Frisch's experimental design to address this question as it represents the most naturalistic and probably a more challenging situation for the animals compared with the proboscis extension reflex paradigm which has frequently been used with honeybees, particularly to assess mechanisms of odor learning (Menzel and Müller, 1996; Menzel *et al.*, 1996). Training free-flying bees to odors also allows simultaneous testing of many odors, thus avoiding the problem of a sequential test procedure as in the proboscis extension preparation.

The aims of the study were threefold: (i) to provide systematic data on the olfactory discrimination ability of free-flying honeybees for an array of substances of varying structural similarity; (ii) to assess whether any correlations exist between discrimination performance and different structural features of the odorants under investigation; and (iii) by comparing our data with those of earlier studies which employed the same odorants with mammals, to assess whether the similiarities found in the structural organization of the olfactory systems of insects and vertebrates are paralleled by similarities in relative discrimination abilities.

Materials and methods

Animals

A total of 78 free-flying honeybee workers (*Apis mellifera carnica*) from one of the colonies maintained at the apiary of the Department of Neurobiology at the Free University of Berlin was used. The hive was 30 m distant from the room in which the experiments were conducted.

Odorants

A total of 44 odorants, including homologous series of aliphatic alcohols, ketones and aldehydes (C4–C10 respectively) was used (Table 1). Further, the set of substances included isomeric forms of aliphatic alcohols and ketones, several terpenes and etheric oils, and one aliphatic ester.

Table 1 Substances used

Alcohols		Aldehydes	
1-Butanol	$C_4H_{10}O$	*n*-butanal	C_4H_8O
1-Pentanol	$C_5H_{12}O$	*n*-pentanal	$C_5H_{10}O$
1-Hexanol	$C_6H_{14}O$	*n*-hexanal	$C_6H_{12}O$
1-Heptanol	$C_7H_{16}O$	*n*-heptanal	$C_7H_{14}O$
1-Octanol	$C_8H_{18}O$	*n*-octanal	$C_8H_{16}O$
1-Nonanol	$C_9H_{20}O$	*n*-nonanal	$C_9H_{18}O$
1-Decanol	$C_{10}H_{22}O$	*n*-decanal	$C_{10}H_{20}O$
2-Pentanol	$C_5H_{12}O$		
2-Hexanol	$C_6H_{14}O$	Terpenes	
2-Heptanol	$C_7H_{16}O$	(+)-limonene	$C_{10}H_{16}$
2-Octanol	$C_8H_{18}O$	(–)-limonene	$C_{10}H_{16}$
2-Nonanol	$C_9H_{20}O$	(+)-menthol	$C_{10}H_{20}O$
3-Pentanol	$C_5H_{12}O$	(–)-menthol	$C_{10}H_{20}O$
1,5-Pentandiol	$C_5H_{12}O_2$	(+)-carvone	$C_{10}H_{16}O$
		(–)-carvone	$C_{10}H_{16}O$
Ketones		linalool	$C_{10}H_{18}O$
2-Butanone	C_4H_8O	geraniol	$C_{10}H_{18}O$
2-Pentanone	$C_5H_{10}O$	citral	$C_{10}H_{16}O$
2-Hexanone	$C_6H_{12}O$		
2-Heptanone	$C_7H_{14}O$	Others	
2-Octanone	$C_8H_{16}O$	isoamyl acetate	$C_7H_{14}O_2$
2-Nonanone	$C_9H_{18}O$	peppermint oil	
2-Decanone	$C_{10}H_{20}O$	orange oil	
3-Heptanone	$C_7H_{14}O$	lime-blossom oil	
4-Heptanone	$C_7H_{14}O$	clove oil	

The rationale for choosing the substances was to present the bees with a series of compounds of varying degrees of structural similarity to any conditioning odorant. Thus, for example, when conditioned with an aliphatic alcohol, the bee was also presented with aliphatic alcohols of different carbon chain lengths (i.e. other members of the homologous series), aliphatic alcohols with the same or similar carbon chain length but a different position of the functional group (isomeric forms), substances with the same or similar carbon chain length but different oxygen moieties (aliphatic aldehydes and ketones), odorants with the same functional group but a cylic structure (terpene alcohols) as well as etheric oils, i.e. complex mixtures which comprise compounds belonging to the same chemical class as the conditioning stimulus.

Most of the substances are known to be components of floral scents (Knudsen *et al.*, 1993), and several of the odorants (geraniol, citral, isoamyl acetate, 2-heptanone and 2-nonanol) have also been identified as components of honeybee pheromones (Free, 1987).

Experimental procedure

The odor stimuli were presented in opaque 1 l glass bottles.

Each bottle contained a circular filter paper with a diameter of 6 cm which was impregnated with 4 µl of an odorant (Table 1). The neck of each bottle was equipped with a tight-fitting Plexiglas tube of 3 cm length and 1.6 cm inner diameter which had a wire mesh at its inner end in order to allow an odor stimulus to emanate from a bottle but to prevent an animal from crawling or flying into the distal part of the bottle.

A total of 48 bottles was arranged horizontally in a rack of 70 × 80 cm in six rows of eight bottles each. To prevent the odors from intermingling in front of the bottles, the rear of the rack was made conical with the center connected to a suction pump which provided a constant and approximately laminar airstream around each bottle neck.

Foraging worker honeybees were trained to approach a feeder baited with a 20% (w/w) sucrose solution situated in proximity to the test apparatus. Regularly returning bees were individually marked, collected from the feeder when approaching it by use of a glass vial, and put into one of the bottles of the apparatus which contained the conditioning odor (S+) and was baited with 60 µl of a 30% (w/w) sucrose solution placed in the neck of the bottle. Usually the bee entered the neck of the bottle voluntarily, ingested the food reward while perceiving the conditioning odor and then returned to its hive. Five such training trials were conducted in order to allow the animal to build a robust association between the food reward and the conditioned odor while taking care to change the position of the S+ bottle in the array between each trial to prevent the occurrence of positional preferences. Following the fifth training trial, the feeder was removed, the position of the bottle containing the conditioned odor was changed again, and on returning from its hive the bee approached the apparatus and started to search for the S+ by hovering in front of the bottle necks. The behavior of the bee was recorded both on videotape and on protocol sheets which showed the position of each stimulus.

Correct choices consisted of animals both landing in the neck of a bottle containing the S+ and failing to land at a bottle containing an S– after hovering in front of the bottle neck for a minimum of 0.5 s. Conversely, errors consisted of landing in the neck of a bottle containing an S– and failing to land at an S+ bottle after hovering in front of the bottle neck for a minimum of 0.5 s.

In order to obtain a sufficient number of decisions, each bee received a total of 10 extinction trials without food reward and 10 food-rewarded test trials which were alternated with the former. In the extinction trials, the array was composed of 43 bottles containing one S– each, one bottle containing the S+ (without a food reward) and four blank bottles without any odor. In the test trials, the array was again composed of the 43 bottles containing one S– each, but with three bottles containing the S+ (with a food reward) and two blank bottles. In order to maximize the number of decisions per bout and to prevent animals from developing a position preference, the food reward in the test trials was restricted to 10 µl of sugar solution (a full crop load of a honeybee is ~60 µl), and an S+ bottle which already had been visited by a bee was only baited again during the same bout when the animal had landed at one of the other two S+ bottles and consumed the food reward provided there. In order to prevent demotivation of the animals, the time allotted to extinction trials 2–10 was restricted to 3 min. The first extinction trial, i.e. the very first trial following the five (food-rewarded) conditioning trials, lasted only 2 min as a longer duration occasionally caused animals to quit searching and reliably returning to the apparatus. Between any of the 20 trials, the arrangement of the bottles was changed according to a pseudo-randomized scheme.

With the exception of two substances [linalool and (–)-limonene], all odorants were used as conditioned stimuli, and with the exception of six substances (*n*-nonanal, *n*-decanal, geraniol, citral, lime-blossom oil and clove oil), data from two bees per S+ were recorded (cf. Figure 1). Usually two bees were tested per day while taking care to renew the odor stimuli and to thoroughly clean the Plexiglas tubes in the neck of the bottles for each animal.

Data analysis

In assessing performance of the bees, only unequivocal decisions (see above) were scored. The minimum number of decisions per animal was 1300, the mean 1708. Significance levels were determined separately for extinction and test trials by calculating binomial *z*-scores corrected for continuity (Siegel and Castellan, 1988) from the number of correct and false responses for each individual and stimulus.

Comparisons across tasks were made using Friedman's two-way ANOVA. When differences between tasks were detected, pairwise Wilcoxon's signed-rank tests were applied for related samples to evaluate which tasks were responsible. Correlations between discrimination performance and structural similarity in terms of differences in carbon chain length were evaluated using Spearman's rank correlation coefficient and tested for significance by computing *t*-values. All tests were two-tailed, and the alpha level was set at 0.05. All data are reported as means ± SD.

Results

General discrimination performance

Figure 1 summarizes the discrimination performance of the 78 honeybees tested in the extinction trials. Of the 1848 odor pairs, the bees were able to significantly discriminate 1793, i.e. 97.0%. Only seven odor pairs (i.e. 0.4% of all odor pairs) were not distinguished by both bees trained to a given S+, and 48 odor pairs (2.6% of all odor pairs) were confused by one bee.

When used as the conditioned stimulus (S+), 15 substances were significantly discriminated from all other

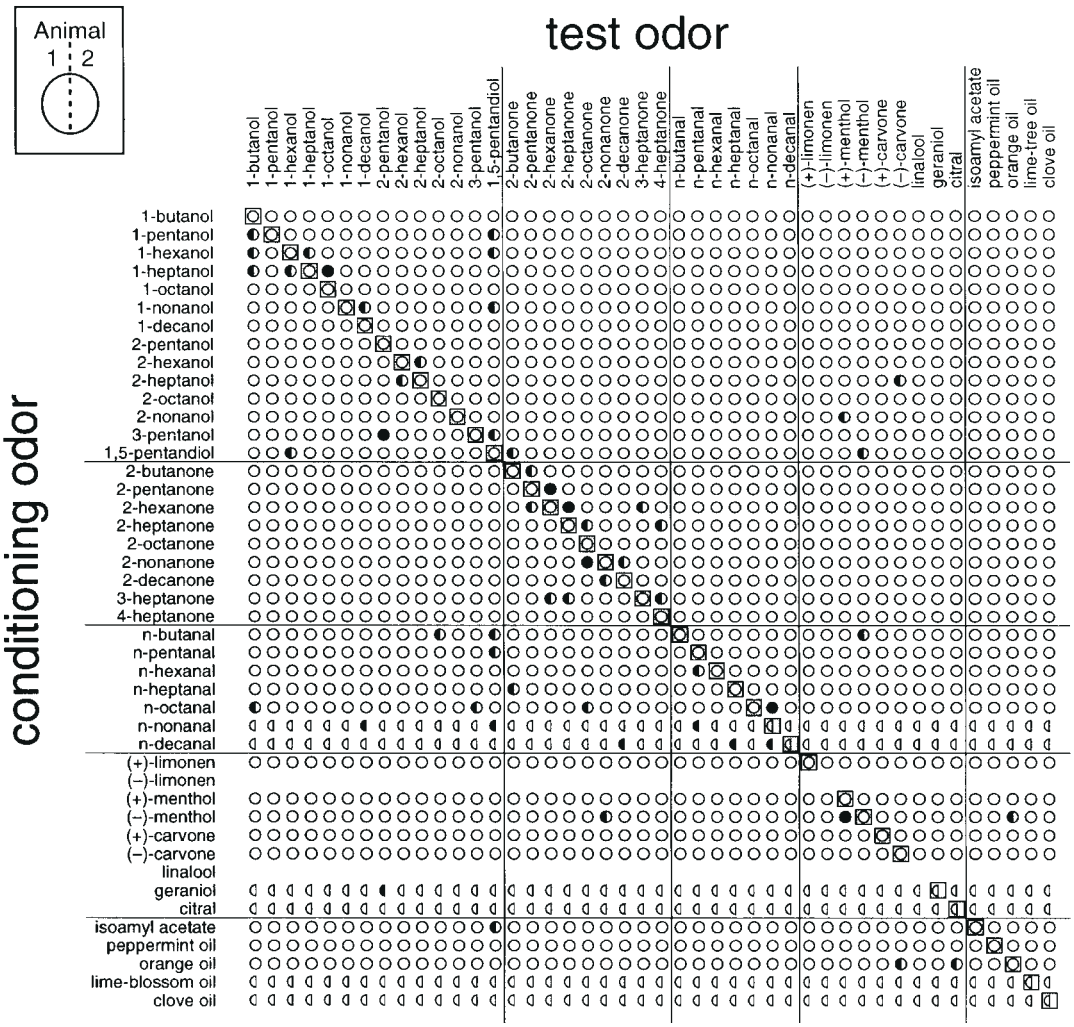

Figure 1 Performance of the 78 honeybees in discriminating between the 44 odorants used. Each circle represents the performance of two honeybees conditioned to the same S+ (see insert in the upper left hand corner). Accordingly, half-circles represent the performance of individual honeybees. Filled areas indicate an animal's failure to significantly discriminate its conditioning odor from another stimulus (two-tailed binomial test, $P < 0.05$).

stimuli, 10 substances were confused with only one of the 43 S–, seven substances with two test odors, nine substances with three, and one substance with four. Thus, all 42 substances employed as S+ were significantly distinguished from >90% of the other stimuli.

Overall discrimination scores in test trials and extinction trials with a given animal were usually very similar and differences between the two only rarely exceeded 5%. Inter-individual variability in overall discrimination scores was markedly low, with all 78 bees scoring >90% correct choices in both the extinction trials and the test trials, and the majority of animals even scoring >95% correct. None of the bees failed to correctly identify its conditioning odor at a significant level (diagonal in Figure 1) and none of the bees confused >4 of the 44 odor pairs.

Carbon chain length

Figure 2 summarizes the discrimination performance of the honeybees as a function of differences in carbon chain length. In all four homologous series of substances employed (2-ketones, *n*-aldehydes, 1-alcohols and 2-alcohols) we found that odor pairs which differed by only one carbon atom were significantly more difficult to discriminate than odor pairs which differed by two or more carbon atoms (Wilcoxon, $P < 0.01$ for all pairs and all four homologous series). Odor pairs which differed by two or more carbon atoms did not differ significantly from each other in their degree of discriminability (Wilcoxon, $P > 0.05$ for all pairs and all four homologous series). Accordingly, a highly significant negative correlation between discrimination performance and structural similarity of odorants in terms of differences in carbon chain length was found in all four homologous series (Spearman, $P < 0.01$). This finding is also illustrated by the fact that 23 out of 27 cases of failure to significantly discriminate between members of a homologous series were due to odor pairs which differed by only

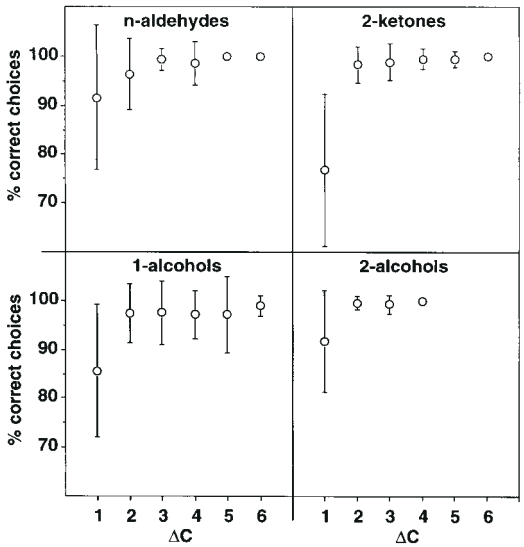

Figure 2 Discrimination performance (means ± SD) in the extinction trials as a function of differences in carbon chain length. ΔC1 corresponds to the discrimination of members of a homologous series of substances which differ by only one carbon atom, and ΔC2 to ΔC6 to the discrimination of substances which differ by two to six carbon atoms respectively (cf. Table 1).

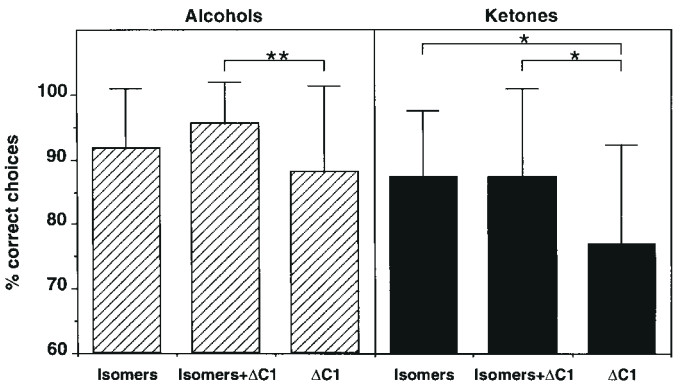

Figure 3 Performance (means ± SD) in the extinction trials in discriminating between substances which differ only in the position of the functional group (isomers), both in position of the functional group and in carbon chain length (isomers + ΔC1), or in carbon chain length only (ΔC1). Left panel: alcohols; right panel: ketones. $*P < 0.05$; $**P < 0.01$.

one carbon atom (cf. Figure 1). The number of cases in which an animal failed to significantly distinguish its conditioned odor from a member of the same homologous series with a shorter carbon chain was 13—almost identical to the 14 cases in which the carbon chain of the unconditioned odor was longer than the S+.

Position of a functional group

The discrimination of an aliphatic alcohol from other stimuli with the same carbon chain length but a different position of the functional alcohol group, i.e. isomeric forms, presented little difficulty to the bees. None of the animals confused any 1-alcohol with a 2-alcohol having the same number of carbon atoms (20 cases), irrespective of whether the conditioned stimulus was an alcohol with a terminal oxygen moiety or an alcohol with a non-terminal alcohol group. The only two cases of failure to significantly discriminate between isomeric forms of aliphatic alcohols occurred with the odor pair 3-pentanol versus 2-pentanol, i.e. two aliphatic alcohols which share a non-terminal alcohol group (cf. Figure 1).

Figure 3 illustrates that the degree of discriminability in odor pairs involving isomeric alcohols, i.e. substances which only differ in the position of the functional group, was at least slightly lower than in odor pairs which not only differed in the position of the functional alcohol group but also differed by one carbon atom. The discriminability of isomeric alcohols was slightly higher, however, than that of alcohols which share the position of the functional group but differ in chain length by one carbon atom. The latter were significantly more difficult to distinguish than isomers that differ by one carbon atom (Wilcoxon, $P < 0.01$).

The discrimination of an aliphatic ketone with seven carbon atoms from other straight-chain heptanones with a different position of the functional keto group led to seven cases of significant discrimination and three cases of failure (2-heptanone versus 4-heptanone, 3-heptanone versus 2-heptanone and 3-heptanone versus 4-heptanone), and thus to a markedly higher proportion of confusions compared with the alcohols (cf. Figure 1).

Figure 3 shows that the degree of discriminability in odor pairs involving isomeric ketones, i.e. substances which differ only in the position of the functional group, was as high as in odor pairs which differed not only in the position of the functional keto group but also by one carbon atom. The discriminability of isomeric ketones was significantly higher, however, than that of ketones which share the position of the functional group but differ in chain length by one carbon atom (Wilcoxon, $P < 0.05$).

The discrimination of a non-aliphatic substance, the terpene alcohol geraniol, from its isomer linalool was readily accomplished.

Number of functional groups

1,5-Pentandiol, an aliphatic alcohol with five carbon atoms and functional alcohol groups at both ends of the carbon chain, presented some difficulties for the bees. This substance was confused with several alcohols of the same or similar carbon chain length, whether assigned as S+ or as S–: there were two cases of failure to discriminate 1,5-pentandiol from 1-hexanol, and one case each with 1-pentanol, 1-nonanol and 3-pentanol (cf. Figure 1).

Type of functional group

The discrimination of aliphatic substances with the same carbon chain length but different oxygen moieties presented

little difficulty to the bees. With 1-alcohols, 2-alcohols and ketones used as S+, there were no cases of confusion with odorants sharing the same number of carbon atoms but differing in their respective functional groups (cf. Figure 1). With the exception of one odor pair (1,5-pentandiol versus 2-butanone) there were even no cases of confusion between aliphatic alcohols (used as S+) and any aliphatic ketone or aldehyde at all. Similarly, 2-ketones (used as S+) were never confused with any aliphatic alcohol or aldehyde (cf. Figure 1).

With *n*-aldehydes used as S+, there were two cases of failure to significantly discriminate between odors on the basis of different oxygen moieties only (*n*-octanal versus 2-octanone and *n*-decanal versus 2-decanone). Additionally, there were three cases of confusing an aliphatic aldehyde with 1,5-pentandiol (versus *n*-butanal, *n*-pentanal and *n*-nonanal used as S+ respectively) and five cases with alcohols or ketones having a different number of carbon atoms compared with the S+ (*n*-butanal versus 2-octanol, *n*-octanal versus 1-butanol, *n*-octanal versus 3-pentanol, *n*-nonanal versus 1-decanol and *n*-heptanal versus 2-butanone). Thus, with the aliphatic aldehydes (used as S+) there were more cases of failure to discriminate between substances with different functional groups ($n = 10$) than cases of failure to distinguish between aldehydes, i.e. substances with the same functional group ($n = 6$).

The discrimination of non-aliphatic substances that are identical in structure except for their oxygen moieties was readily accomplished by all bees. Geraniol (a terpene alcohol), for example, was never confused with citral (syn. geranial, the corresponding terpene aldehyde), and similarly carvone (a terpene ketone) was easily discriminated from limonene (syn. carvene, the corresponding terpene hydrocarbon), irrespective of which member of these odor pairs was used as S+ or as S–.

Cyclic substances

The discrimination of aliphatic substances from cyclic substances led to only a few cases of confusion. Three cases concerned odor pairs which share an alcohol group as their oxygen moieties [2-nonanol versus (+)-menthol, geraniol versus 2-pentanol and 1,5-pentandiol versus (–)-menthol] and three cases involved odor pairs that differ in their functional groups [2-heptanol versus (–)-carvone, *n*-butanal versus (–)-menthol and (–)-menthol versus 2-nonanone].

The only two cases of failure to discriminate between two cyclic substances concerned a pair of enantiomers, (–)-menthol versus (+)-menthol, i.e. two substances that are identical in molecular structure except for chirality. Interestingly, the bees did not confuse the other two enantiomeric odor pairs (the optical antipodes of limonene and of carvone) employed (cf. Figure 1).

Odor mixtures

The discrimination of complex odor mixtures from other odorants again presented little difficulty to the bees. The etheric oils employed were never confused with other multicomponent odorants, irrespective of whether they were assigned as S+ or as S– (cf. Figure 1). The only three cases of failure to significantly discriminate between an odor mixture and a monomolecular substance involved orange oil, which was confused by single bees with citral, (–)-carvone and (–)-menthol respectively. The first two of these three substances are known to be components of orange oil (Nursten, 1970).

Pheromone compounds

Five of the odorants used (geraniol, citral, isoamyl acetate, 2-heptanone and 2-nonanol) have been identified as components of honeybee pheromones (Free, 1987). Four of these substances were only confused with one of the other 43 stimuli [geraniol with 2-pentanol, citral with orange oil, isoamyl acetate with 1,5-pentandiol and 2-nonanol with (+)-menthol]. With the fifth pheromone compound used, 2-heptanone, there were five cases of failure to significantly discriminate this substance from other odorants which all concerned other, structurally similar, ketones (3-heptanone, 4-heptanone, 2-octanone, and two cases with 2-hexanone; cf. Figure 1).

Readiness to accept a conditioned odor

Although not systematically assessed, it was evident that the vast majority of odor stimuli was readily accepted by the bees as their respective S+ in the course of the conditioning procedure. The only two substances which presented some difficulties in this respect were aliphatic aldehydes, *n*-nonanal and *n*-decanal. Several bees obstinately refused to leave the glass vials used to take them from the feeder to the bottle containing one of these substances when assigned as S+ (cf. Materials and methods) and to enter the neck of the bottle, and thus had to be replaced by conspecifics.

Training effects

Figure 4 shows the discrimination performance of the bees as a function of the number of test and extinction trials respectively. Performance in terms of number of errors per trial improved systematically across trials and thus a significant training effect at the group level was found (Spearman, $P < 0.05$). Interestingly, the mean number of errors in a given trial did not differ significantly between test and extinction trials (Wilcoxon, $P > 0.05$ in all trials). The fact that the mean number of errors in extinction trial 1 was lower than in extinction trial 2 can be attributed to the shorter duration of the former (cf. Materials and methods).

Discussion

The results of the present study demonstrate (i) that honeybees have an excellent olfactory discrimination ability when tested in a free-flying situation with an array of structurally

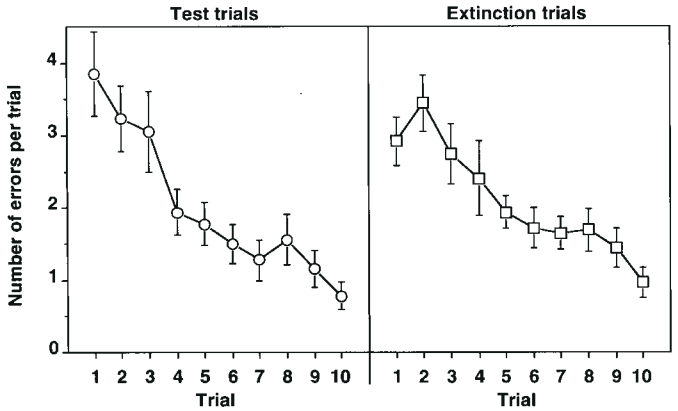

Figure 4 Discrimination performance (mean ± SE) as a function of the number of test trials (left panel) and extinction trials (right panel).

related monomolecular substances; (ii) a significant negative correlation between discrimination performance and structural similarity of odorants in terms of differences in carbon chain length with all substance classes tested; (iii) that both the position and type of a functional group also affected stimulus quality and thus discriminability of odorants, although in a substance class-specific manner; and (iv) striking similarities in odor structure–activity relationships between honeybees and human and nonhuman primates tested previously on a subset of substances employed here.

The excellent performance of *A. mellifera* found here is in agreement with earlier studies on odor discrimination using free-flying honeybees (von Frisch, 1919) or conditioning of the proboscis extension reflex (Vareschi, 1971). However, whereas von Frisch's landmark study mainly employed etheric oils, i.e. complex odor mixtures of high biological significance both in the contexts of nectar source recognition (Pham-Delegue *et al.*, 1993) and nestmate recognition (Bowden *et al.*, 1998), we could show that honeybees are also able to clearly distinguish between a large number of monomolecular substances in a paradigm designed to simulate odor-guided foraging behavior. This ability has so far only been shown employing proboscis extension reflex conditioning, which represents a far more artificial test situation than our naturalistic experimental approach, and which has the disadvantage of sequential rather than simultaneous presentation of stimuli. Despite the presumably more challenging conditions of a free-flying test situation, the honeybees in our study scored an average of >95% correct choices and thus showed almost exactly the same performance as in Vareschi's study (95.5% correct choices), which employed restrained animals. It is remarkable that our experimental set-up which required an animal to perform approach flights and spatial orientation in addition to the actual discrimination task, and which exposed the bees to the natural and ever-changing climate obviously did not impair performance.

Odor structure–activity relationships have so far only rarely been tested systematically in honeybees. We found a significant negative correlation between discrimination performance and structural similarity of odorants in terms of differences in carbon chain length with all four homologous series of substances tested (1-alcohols, 2-alcohols, *n*-aldehydes, and 2-ketones; cf. Figure 2). This finding is in line with an earlier report of proboscis extension conditioning with carboxylic acids (Vareschi, 1971). Similar to our findings, direct neighbors in this homologous series were more difficult to distinguish for the bees than substance class members that differed by two or more carbon atoms. In contrast to our findings, however, Vareschi reported that carboxylic acids with a shorter carbon chain compared with the conditioned odor led to more confusions than those with a longer carbon chain. More recently, it has been shown that alcohols of similar carbon chain length elicited similar activity patterns in the honeybee antennal lobe as visualized by *in vivo* calcium imaging (Sachse *et al.*, 1998). In an extension of this study (Sachse *et al.*, submitted for publication) the authors found that regular connections between this molecular property and glomerular activation patterns also occur with aliphatic aldehydes, ketones, carboxylic acids and alkanes.

The same correlation between differences in carbon chain length and discriminability as shown here has also been reported in several species of mammals. Discrimination performance in squirrel monkeys and humans was found to vary systematically as a function of structural similarity of aliphatic esters (Laska and Freyer, 1997), carboxylic acids (Laska and Teubner, 1998), 1-alcohols, *n*-aldehydes and 2-ketones (Laska and Teubner, 1999b) (M. Laska *et al.*, submitted for publication). It has also been reported (Linster and Hasselmo, 1998) that rats generalized between aliphatic aldehydes of similar carbon chain length whereas their response to less similar aldehydes was comparable to that for a control odor.

The results of these behavioral studies correspond with electrophysiological findings which showed that tuning specificities of rodent olfactory receptor neurons correlate with carbon chain length of aliphatic alcohols (Hirono *et al.*, 1998; Sato *et al.*, 1994) and aldehydes (Zhao *et al.*, 1998). Similarly, single cell recordings from the rabbit olfactory bulb showed that molecular receptive ranges for both excitation and inhibition of single mitral cells are critically determined by the carbon chain lengths of alcohols (Imamura *et al.*, 1992), aldehydes (Mori, 1995; Yokoi *et al.*, 1995) and ketones (Mori and Yoshihara, 1995).

Thus, both the behavioral and the electrophysiological findings suggest that the carbon chain length of aliphatic odorants—irrespective of their chemical class—is a critical determinant of the interaction between the stimulus molecule and its receptor in both vertebrates and insects.

With regard to the type of functional group, the second molecular feature investigated here, we found that stimulus quality and thus discriminability of odorants was affected in

a substance class-specific manner. Whereas the discrimination of substances with the same or a similar carbon chain length but different oxygen moieties presented no difficulties to the bees when one of the discriminants was an alcohol or a ketone, there were several cases of failure when one of the odors in a given stimulus pair was an aldehyde. This suggests that substances with a functional aldehyde group might interact with a broader range of molecular receptors than substances belonging to one of the other two chemical classes tested.

In an attempt to quantify odor discrimination using electromyogram recordings of the proboscis extension reflex with a smaller set of substances (three esters, four aldehydes, and five ketones and alcohols), it was found (Smith and Menzel, 1989) that alteration of carbon chain length had more of an effect on perceptual similarity for alcohols and ketones than for aldehydes and acetic esters. Our data correspond with this observation as the mean difference in discrimination scores between ΔC1 and ΔC2 odor pairs was larger with alcohols and ketones than with aldehydes (cf. Figure 2).

Based on single-cell recordings from antennal placodes, honeybee olfactory receptor neurons could be assigned to 10 reaction groups which at least coarsely corresponded to different chemical classes (Vareschi, 1971). These findings, however, have been questioned in more recent studies (Akers and Getz, 1992; Getz and Akers, 1993) which failed to confirm Vareschi's results employing more sophisticated recording techniques.

Investigations of human discrimination ability as a function of type of functional group have so far concentrated largely on cyclic rather than aliphatic substances and generally found perceptual similarity to be odor pair-specific, ranging from easily distinguishable odor pairs to indiscriminable ones, with no recognizable correlation between odor quality and this structural property of the stimulus (Ohloff *et al.*, 1991; Yoshii and Hirono, 1996).

Single-cell recordings from olfactory receptor cells in mice (Sato *et al.*, 1994) and from mitral cells in the olfactory bulb of rabbits (Imamura *et al.*, 1992) in response to stimulation with aliphatic substances from different chemical classes, however, suggest functional groups to be an important determinant of tuning specificity and thus response selectivity of mammalian olfactory sensory neurons.

Position of a functional group, the third molecular feature investigated here, was also found to clearly affect odor quality, again in a substance class-specific manner. Whereas the bees had little difficulty in distinguishing between 1-alcohols and 2-alcohols, there were several cases of failure with isomeric forms of ketones. In both classes of substances, however, we found discrimination scores for isomeric odor pairs to be higher than for odor pairs sharing the position of their respective functional group but differing in chain length by one carbon atom (cf. Figure 3). This suggests that odor quality of aliphatic carbohydrons as perceived by honeybees is also critically determined by the position of functional alcohol and keto groups. Similar findings in human and nonhuman primates, which both readily discriminated between isomeric forms of alcohols and ketones, and performed better in these tasks compared with discriminations involving non-isomeric odor pairs which differed by one carbon atom (M. Laska *et al.*, submitted for publication), again indicate that similar, if not identical, mechanisms of odor coding may apply to both vertebrates and insects.

Our finding that the discrimination of cyclic substances from aliphatic substances led to only few cases of confusion concurs with the idea that overall molecular shape is another important determinant of differential interaction of odor stimuli with olfactory receptors (Yoshii and Hirono, 1996). The only cases of failure to discriminate between two cyclic substances concerned the enantiomers of menthol, i.e. substances that are identical in structure except for chirality. The reasons why two other pairs of optical antipodes, limonene and carvone, were readily discriminated by the bees remain to be revealed. It is interesting to note, however, that exactly the same pattern of discrimination performance with these three pairs of enantiomers has been found with human subjects (Laska and Teubner, 1999a). This suggests that even phylogenetically distant species might express the same or at least similar types of enantioselective molecular odor receptors.

It is commonly agreed that the olfactory systems of both vertebrates and insects are particularly adjusted to process complex odor mixtures as the vast majority of naturally occurring odors in our environment comprise numerous compounds (Laing *et al.*, 1989). Our finding that the bees readily discriminated between all etheric oils employed is in line both with this view and with von Frisch's study (von Frisch, 1919). However, the fact that at least some confusions between odor mixtures and monomolecular substances which are known to be constituents of these mixtures occurred suggests that honeybees—similar to mammals (Laska and Hudson, 1993)—may at least partially rely on key compounds, i.e. monomolecular substances that characterize a *Geruchsgestalt*, to recognize odor mixtures. This assumption is supported by recent findings from a coupled gas chromatography–proboscis extension reflex assay that showed honeybees to selectively respond to certain components of an extract of oilseed rape floral volatiles after conditioning to the complete extract (Le Metayer *et al.*, 1997).

A final aspect of the present study is our finding that the discrimination performance of the bees systematically improved across trials (cf. Figure 4). This is remarkable considering that numerous studies have shown that honeybees are capable of one-trial associative learning with odor stimuli (Menzel and Müller, 1996; Menzel *et al.*, 1996), and considering that our experimental procedure even included five conditioning trials in order to allow an animal to build

a robust association between the food reward and the conditioning odor (cf. Materials and methods). The ability to correctly respond to a conditioned odor after a single trial, however, does not necessarily imply that this learning process is sufficient to reliably discriminate between qualitatively similar odors. Rather, it seems that several encounters with the odors in question are necessary to fully exploit the discriminatory power of the olfactory system. Thus, this finding is likely to illustrate the significance of experience for odor discrimination (Rabin, 1988).

Taken together, our findings demonstrate that the similarities found in the structural organization of the olfactory systems of insects and vertebrates are paralleled by striking similarities in relative discrimination abilities. This strongly suggests that similar mechanisms of odor coding and discrimination may underlie olfaction in these two groups of animals. In order to further corroborate this hypothesis and to draw further conclusions as to the nature and generality of odor structure–activity relationships across the animal kingdom, it seems worthwhile to follow a comparative approach including both phylogenetically distant and closely related species, and to test their discrimination abilities using the same sets of stimuli.

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