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COMB-WAX DISCRIMINATION BY HONEYBEES TESTED WITH THE PROBOSCIS EXTENSION REFLEX

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Summary

We used the proboscis extension reflex of honeybees to test their ability to discriminate between comb waxes of different ages (wax scales, 1-week-old wax, 2- to 3-year-old wax, 8- to 10-year-old wax). Such waxes differ in their chemical composition, and an ability to discriminate between them may aid the orientation of the bees in the nest.

To train the bees, we used whole extracts of waxes and four different fractions of the whole extract based on different elutions of solid-phase extractions (extract I, fraction A eluted with hexane and fraction B with diethylether; extract II, fraction B further subdivided into fraction C by elution with isopropylchloride and fraction D by elution with diethylether).

In a differential training regime (six learning and six test trials) with whole extracts or with the different fractions, we paired one type of wax with a reward and another with no reward. The bees learned to discriminate between all tested pairs of whole extracts. The two subfractions (fractions A and B) gave different results: the bees could discriminate between waxes of different ages when fraction B was used but not when fraction A was used. A further subdivision of fraction B into fractions C and D showed that only fraction D contained the elements that enabled bees to discriminate between old and new wax.

Fraction D makes up only 5–8 % of the total wax mass and contains hydroxy alkyl esters (5–6 % of the total wax mass), primary alcohols (0.3–0.5 % of the total wax mass) and acids (0.06–1.0 % of the total wax mass). Fractions A and C (together forming 62–64 % of the total wax mass), which consist of unbranched and branched aliphatic hydrocarbons and alkyl esters, could not be discriminated by the bees. The remaining wax mass (25–29 %) was eluted with a mixture of chloroform, methanol and water (13:5:1) as fraction E.

Key words: honeybee, *Apis mellifera*, comb wax, chemical component, discrimination of wax, proboscis extension reflex.

Introduction

Honeybees are constantly exposed to self-produced wax both in the form of comb wax, which is used as building material for brood and storage cells, and as cuticular wax, which covers the whole surface of each individual. The chemical composition of wax is highly complex and variable (Hepburn, 1986), but comb and cuticular wax have the same qualitative composition: the two differ only in the relative amounts of their components. Waxes of different ages have different compositions (Fröhlich et al., 2000), comb waxes from different colonies vary considerably (Breed et al., 1995a) and cuticular wax has strong colony- and kin-related composition variations (Francis et al., 1985, 1989; Page et al., 1991).

Such systematic compositional variations may provide the honeybees with important information. Cuticular wax cues are the basis for nestmate (Breed and Stiller, 1992) and kin (Getz and Page, 1991) recognition, while additional cues, which may even override the cuticular cues for nestmate recognition, are acquired from comb wax (Breed et al., 1995b). It has been hypothesised that discriminating between comb waxes provides the evolutionary basis for kin recognition (Breed and Stiller, 1992). The ability to discriminate between comb waxes has been demonstrated in a series of behavioural studies. In the hive, bees have a strong tendency to store nectar in older rather than in younger combs (Free, 1987). They are able to discriminate between combs from their own and a different colony (Breed and Stiller, 1992), and in a choice test (olfactometer) they prefer familiar to unfamiliar wax (Breed and Stiller, 1992). Honeybees recognise a delimited comb region as their dance floor even if it is displaced within the nest (Tautz and Lindauer, 1997). The cues responsible for these behavioural achievements may be wax components and/or cues such as the aromatic oils of plants added to the wax (the latter was termed the ‘scented candle’ model by Gamboa et al., 1986).

Here, we focus on wax components as cues for discrimination between comb waxes. The perception of comb waxes may be based both on contact chemoreception and on airborne signals. Honeybees learn to distinguish volatile odours of various chemical classes (acids, alcohols, oxo-acids, ketones, terpenols and flower scents) very efficiently (Vareschi, 1971). Low-volatility compounds (cuticular chemicals of the thorax) can also be differentiated (Getz et al., 1986).

Since wax consists of more than 90 % aliphatic compounds (Hepburn, 1986; Fröhlich et al., 2000) and aliphatic compound profiles show considerable variability (Breed et al., 1995a), research on the ability of insects to discriminate waxes has focused on these compounds, particularly on aliphatic hydrocarbons (for reviews, see Howard, 1993; Smith and Breed, 1995). In a previous study, we used a statistical discriminant analysis to show that the chemical composition of comb waxes of different age classes can be differentiated (Fröhlich et al., 2000).

Here, we tested whether honeybees can distinguish between different comb waxes and, if so, on which chemical wax components their performance is based. As a behavioural paradigm, we used the proboscis extension reflex, a tool that has been used extensively and successfully in studying learning and sensory discrimination in bees (Hammer and Menzel, 1995).

Materials and methods

Waxes

Samples of comb wax were taken in October 1996 from a 20-frame hive of *Apis mellifera carnica* Pollm. from frames of known age. Comb wax belonging to three age classes was obtained: (i) 1 week old (designated 'new wax'), (ii) 2–3 years old ('middle-aged wax') and (iii) 8–10 years old wax ('old wax'). Wax scales (WS) secreted from the wax glands on the abdomen of the bees were collected from the bottom of the same hive immediately after they had fallen off the bees. The wax scales and comb waxes were dissolved in chloroform ($CHCl_3$; purity >99.9 %; C. Roth, Karlsruhe, Germany) at a concentration of 1 mg ml^{-1}.

Two wax fractions (extract I) were obtained using a solid-phase extraction method described previously in detail (Nass et al., 1998) by elution with hexane (fraction A) and diethylether (fraction B). Subsequently, three fractions (extract II) were obtained by elution with hexane (fraction A), isopropylchloride (fraction C) and diethylether (fraction D). Preliminary tests had shown that another fraction (fraction E) could be eluted with a mixture of chloroform, methanol and water (13:5:1), but this contained the most polar constituents of the wax and was not directly amenable to gas chromatography and mass spectrometry and, therefore, was not studied further.

Qualitative and quantitative analysis

Fractions A–D resulting from solid-phase extraction of the raw wax extracts were amenable to gas chromatographic analysis. For this purpose, the hydroxyl and carboxyl groups of the wax components present in fractions B and D were transformed to the corresponding trimethylsilyl derivatives by *N*,*O*-bis-(trimethylsilyl)-trifluoroacetamide (BSTFA; Macherey-Nagel, Düren, Germany) in pyridine (purity >99.5 %; E. Merck, Darmstadt, Germany) for 30 min at 70 °C.

Capillary gas chromatography (5890 series II, Hewlett Packard, Avondale, PA, USA) with on-column injection onto a 30 m DB-1 open tubular fused silica column (internal diameter 0.32 mm, film 0.1 μm; J&W Scientific, Folsom, CA, USA) and flame ionisation (FI) or mass-selective detection (MS; 70 eV, *m*/*z* 50–650, where *m* is mass and *z* is valence; Hewlett-Packard 5971) were carried out using the following temperature programme: injection at 50 °C, 2 min at 50 °C, 40 °C min^{-1} up to 200 °C, 2 min at 200 °C, 3 °C min^{-1} up to 320 °C (FI) or 300 °C (MS), 30 min at 320 °C (300 °C). Carrier gas pressures were adjusted as follows: 30 min at 50 kPa, 10 kPa min^{-1} up to 150 kPa for flame ionisation detection (hydrogen) and 30 min at 10 kPa, 10 kPa min^{-1} up to 100 kPa for mass-selective detection (helium). For the quantification of wax components, internal standards were added to the raw extracts. Tetracosane, methyl triacontanoate and tetracosanoic acid were chosen as standards because they co-eluted with each of the wax fractions analysed by gas chromatography.

The waxes and the honeybees tested were taken from different hives. For the conditioning of the proboscis extension reflex, the wax solutions were applied to glass rods (100–200 μg per rod), and the solvent was allowed to evaporate until dry. Subsequently, the rods were heated to 90 °C for 3 min (to make a smooth surface identical for all probes and thus not to giving any mechanical cue for discrimination) and then stored at −18 °C for up to 2 weeks until used in experiments.

Conditioning

Differential conditioning of the proboscis extension reflex followed a slightly modified basic method described previously in detail (Menzel et al., 1993). Test bees were collected at the hive entrance and harnessed so that only their mouth parts and antennae could move freely. Glucose stimulation to the antennae as an unconditioned stimulus

Table 1. *Learning and testing scheme according to which the conditioning was performed*

Phase	Trial					
Learning						
Trial	1	2	3	4	5	6
CS	+	–	–	+	–	+
US	+	–	–	+	–	+
Testing						
Trial	7	8	9	10	11	12
CS	+	–	–	+	–	+

CS, conditioning stimulus; US, unconditioned stimulus; +, reward with sugar water; –, no reward.

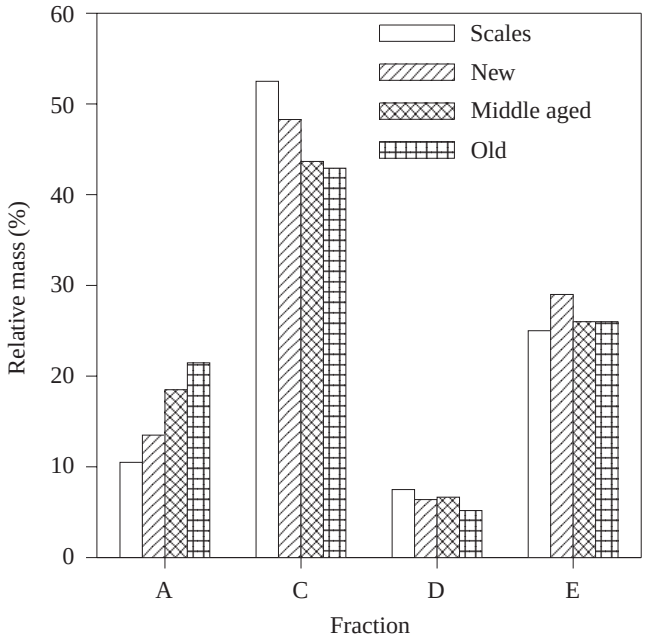

Fig. 1. Analytical yields deriving from the analysis of fractions A, C, D (C+D=B) and E of different-aged comb waxes of *Apis mellifera carnica*.

(US) will elicit proboscis extension as an unconditioned response, but if a wax surface on a glass rod as the conditioned stimulus (CS) is properly paired with the unconditioned stimulus, the wax stimulus itself can elicit the proboscis extension as a conditioned response. Furthermore, if one wax is the positive conditioned stimulus (CS+) and a second wax is the stimulus without reward (CS–), bees stimulated by CS+ should respond with a proboscis extension reflex and should show no reaction when stimulated by CS– if they are able to discriminate between these two stimuli. Waxes from the same solutions on two different rods were used as a non-learning control. The conditioning was performed according to the learning and testing scheme described in Table 1. For each experiment, two repetitions were performed with 20 bees. Three repetitions were performed for the control of extract I and seven for the control of extract II. Bees that reacted spontaneously with proboscis extension in the first trial of the learning phase were omitted from the analysis.

Table 3. *Summary of the results of learning experiments with whole extracts of waxes using different combinations of CS+ and CS– waxes*

		Old wax		Middle-aged wax		New wax	
		+	–	+	–	+	–
Wax scales	+		34 (*N*) 2.17 (Z_e)		38 2.10		37 1.86
	–	28 1.33		38 1.88		37 0.60	
New wax	+		34 1.25		36 1.32		
	–	**34** **1.00**		36 2.25			
Middle-aged wax	+		33 1.92				
	–	31 2.33					

CS+, with reward; CS–, no reward.

The bold numbers correspond to Fig. 3A. All pairs give significantly different results.

Upper value of each pair, number of bees tested (*N*); lower value, median of errors (Z_e) for each combination of waxes (control data: *N*=50, Z_e=2.99 shown in Fig. 3B).

Data analysis

Following Getz et al. (1986), the numbers of errors per bee in the testing phase were calculated, frequency histograms of the numbers of individuals that made 0–6 errors were constructed for each group of 35–40 test bees, and the medians

Table 2. *Relative chemical composition of different fractions of honeybee comb waxes of different ages*

Fraction	Substance class	Relative mass (%)[a]			
		Wax scales	New wax	Middle-aged wax	Old wax
A	Alkanes	11±4.9	13±1.7	15±1.7	14±1.1
	Alkenes	3.4±1.43	6.0±1.04	8.8±0.98	12±1.3
	Alkadienes	0.06±0.044	0.24±0.041	0.72±0.077	2.0±0.21
	2-Methyl alkanes	0±0.008	0.19±0.117	0.46±0.053	0.95±0.129
B C	Alkyl esters	57±6.9	57±3.6	47±4.0	48±4.3
	Unsaturated alkyl esters	13±3.3	11±0.7	12±1.4	9.5±1.54
D	Hydroxy alkyl esters	8.0±3.08	7.9±5.72	8.1±1.57	6.4±0.98
	Acids	1.3±2.00	0.14±0.158	0.51±0.338	0.08±0.105
	Alcohols	0.41±0.239	0.53±0.317	0.74±0.128	0.48±0.202
	Unidentified	5.6±2.97	4.2±2.99	6.2±2.59	7.3±2.03

[a]The values given are related to the total mass of fractions A–D. Values are means ±95 % confidence intervals (*N*=6).

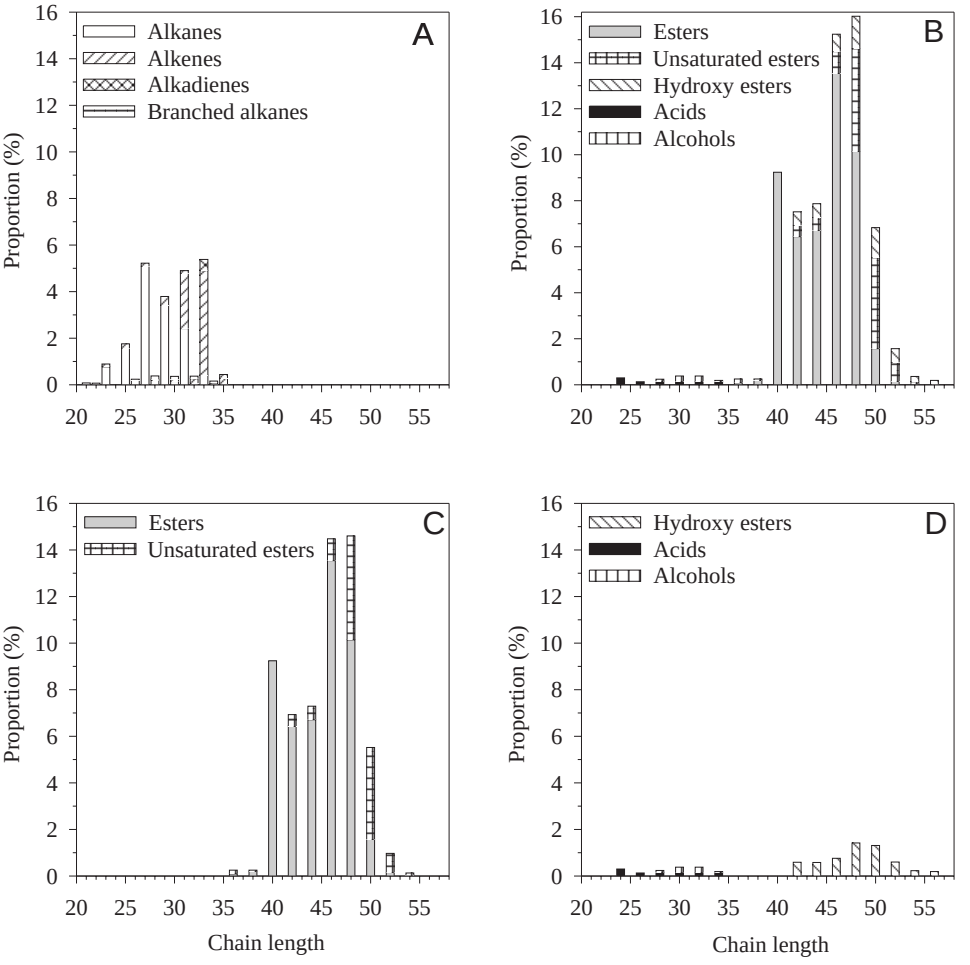

Fig. 2. Relative chemical compositions and distribution of chain lengths of fractions A–D (graphs A–D respectively) of middle-aged comb wax of *Apis mellifera carnica*.

of errors were calculated. χ^2-tests were conducted to compare the error histograms of the tests with the control experiments. To minimise the risk of Type I error, the following significance levels were chosen: significant if $P \leqslant 0.0001$; not significant if $P > 0.0001$. If there was a significant difference between the experiment and the control, it was evident that the bees could discriminate between the waxes.

Results

Chemical analysis

The major components of whole extracts of comb waxes of different ages were alkyl esters, unsaturated alkyl esters, alkanes, hydroxy alkyl esters and alkenes. The minor components were alkadienes, 2-methyl alkanes, acids and alcohols (Table 2). More detailed results are presented in an earlier study (Fröhlich et al., 2000).

As shown in Figs 1 and 2, fraction A made up 11–21 % of the total wax mass and was composed of alkanes, alkenes, alkadienes and 2-methyl alkanes. This fraction was equivalent to the hexane-extractable branched and unbranched aliphatic hydrocarbons of most studies dealing with kin or nestmate recognition by honeybees. Fraction B consisted of alkyl esters, unsaturated alkyl esters, hydroxy alkyl esters, acids and primary alcohols (48–60 % of the total

Table 4. *Summary of results of learning experiments with extract I and fractions A and B of comb waxes using different combinations of CS+ and CS– waxes*

		Old		Middle		Scales	
		+	–	+	–	+	–
Fraction A							
New	+		36 (*N*) 2.80 (Z_e) NS		39 2.73 NS		40 2.68 NS
	–	37 2.6 NS		35 2.77 NS		37 2.84 NS	
Fraction B		+	–	+	–	+	–
New	+		39 0.75		36 1.30		37 1.25
	–	**38** **1.00**		37 1.31		38 1.92	

CS+, with reward; CS–, no reward.

Values are given as the number of bees tested (*N*) and the median of errors (Z_e) for each combination of waxes (control data: N=50, Z_e=2.99).

NS, not significant.

The bold numbers correspond to Fig. 3D.

wax mass). Fraction B was further subdivided into two additional fractions: fraction C consisted of alkyl esters and unsaturated alkyl esters, with 43–53 % of the total wax mass, and fraction D, composed of hydroxy alkyl esters, acids and primary alcohols, made up 5–8 % of the total wax mass. A further fraction (fraction E) that eluted with a mixture of chloroform, methanol and water (13:5:1) was not directly amenable to gas chromatography (25–29 % of the total wax mass).

The whole extract and fraction A can be discriminated significantly (P<0.0001) into the different age classes using discriminant function analyses. On the basis of fractions B, C and D, the waxes can be significantly discriminated on the basis of single components (see also Fröhlich et al., 2000).

Classical conditioning

Bees could discriminate significantly (P<0.0001) between different age classes of comb waxes using whole extracts (Table 3; Fig. 3A). When tested with extract I, the bees did not discriminate between waxes on the basis of the components of fraction A (Fig. 3C), but their discrimination ability on the basis of the components of fraction B was as good as their discrimination with the whole extracts (Table 4; Fig. 3D). When tested with extract II, the bees again did not discriminate between waxes using fractions A and C (Table 5; Fig. 3C,E), but they were able to discriminate on the basis of fraction D (Table 5; Fig. 3F). In the control experiments for extracts I and II (testing for any unknown bias in the experiments), the bees could not discriminate between identical waxes on different rods (Fig. 3B).

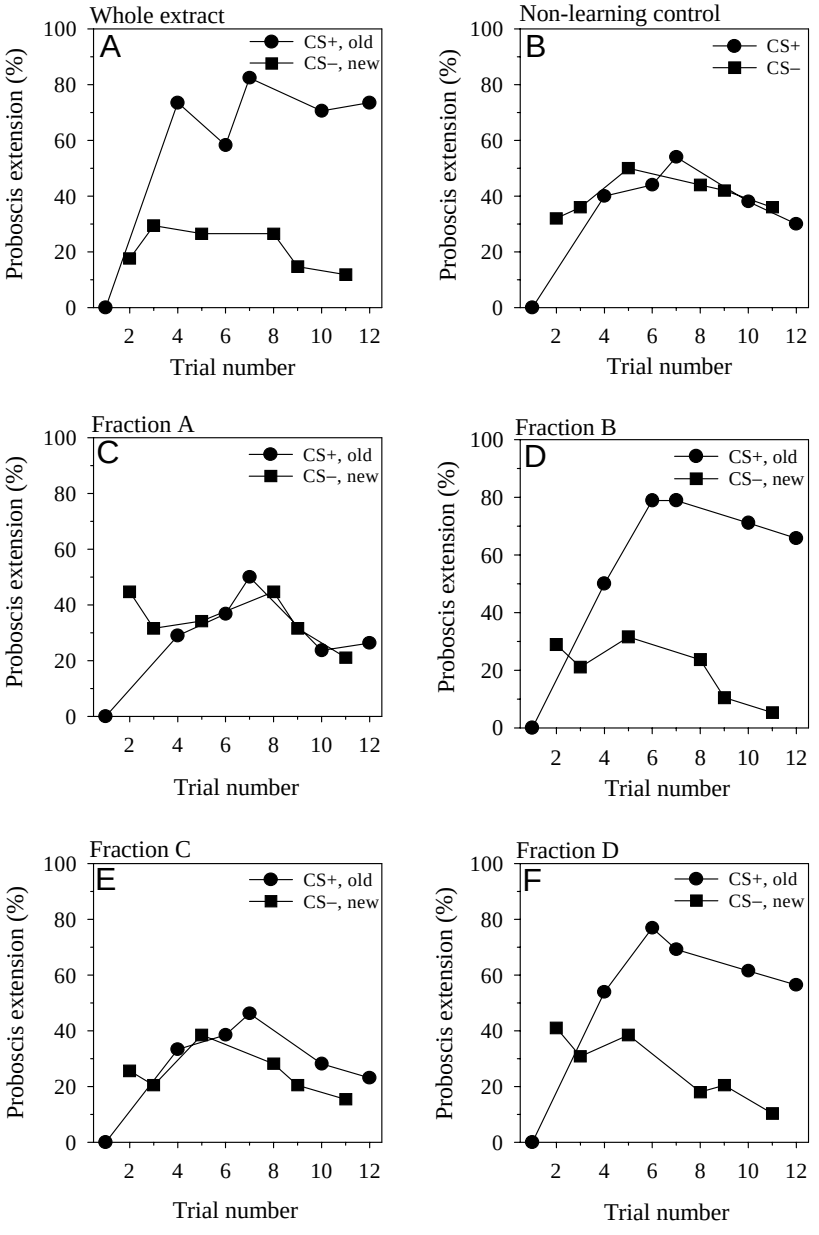

Fig. 3. Discrimination curves (N=20+20 bees for each plot) resulting from differential conditioning of the proboscis extension reflex of *Apis mellifera carnica*. Two different comb waxes were tested using whole extracts and fractions A–D. (A,D,F) Bees are able to discriminate between waxes; (B,C,E) bees cannot discriminate between waxes. Trials 1–6, learning; trials 7–12, testing (see Table 1).

Table 5. *Summary of results of learning experiments with extract II and fractions A, C and D of old and new comb waxes using different combinations of CS+ and CS– waxes*

	Fraction A		Fraction C		Fraction D	
	Old +	Old –	Old +	Old –	Old +	Old –
New +		38 (*N*) 2.90 (Z_e) NS		57 2.99 NS		39 2.69
New –	**38** **2.96** **NS**		**57** **2.83** **NS**		**39** **1.58**	

CS+, with reward; CS–, no reward.

Bold numbers corresponds to Fig. 3C for fraction A, to Fig. 3E for fraction C and to Fig. 3F for fraction D.

Values are given as the number of bees tested (*N*, upper numbers) and the median of errors (Z_e, lower numbers) for each combination of waxes (control data: N=125, Z_e=2.96).

NS, not significant.

Table 6. *Learning experiments with fraction A from extracts I and II of different-aged comb waxes*

Extract	CS+	CS–	*N*	Z_e	*P*
I	Scales	New	37	2.84	NS
I	New	Scales	40	2.68	0.0001
I	New	Middle	39	2.73	0.01
I	Middle	New	35	2.77	NS
I	New	Old	36	2.80	0.05
I	Old	New	37	2.68	0.05
II	New	Old	38	2.90	NS
II	Old	New	38	2.96	NS

Different combinations of CS+ (with reward) and CS– (with no reward) waxes, number of tested bees (*N*), median of errors (Z_e) and error probability (*P*) for each combination of waxes.

NS, not significant.

Discussion

Using the conditioned proboscis extension reflex, the bees learned to react to each wax and each comb wax fraction equally well, because each wax and each fraction was used and tested both as a positive and as a negative conditioning stimulus. However, the distinction between different waxes is not based on aliphatic hydrocarbons since fraction D, containing only hydroxy alkyl esters, primary alcohols and acids, enabled the bees to learn the difference between comb waxes (Fig. 4). This agrees with the work of Vareschi (1971), who showed that bees can learn the odours of polar substance classes (for example, C4–C14 acids and C5–C10 alcohols) very well. A further division of fraction D by thin-layer chromatography into its three substance classes and subsequent conditioning of the proboscis extension reflex should reveal which substance class provides the information for the honeybees.

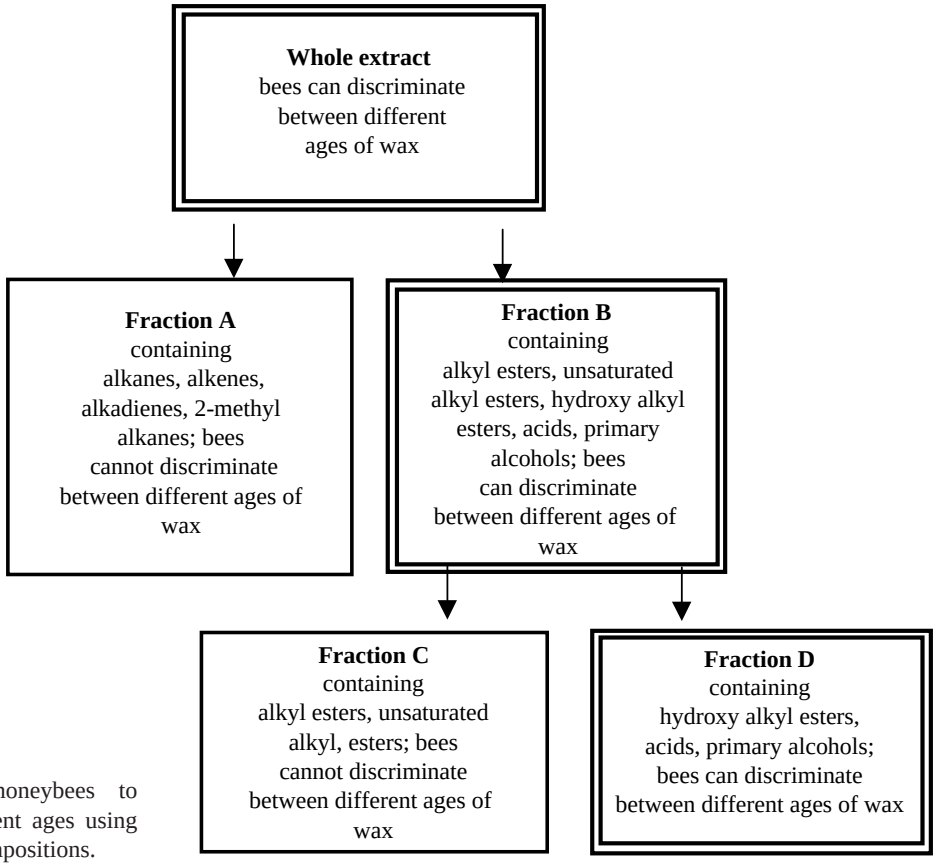

Fig. 4. Illustration of the ability of honeybees to discriminate between comb waxes of different ages using different fractions with different chemical compositions.

The ability of honeybees to discriminate between odours and compounds that are chemically very similar is well developed (Getz and Smith, 1987). They are even able to discriminate between different blends of tricosane and pentacosane and between different blends of undecanoic and dodecanoic acids. The obvious differences in the alkadienes and alkenes of the aliphatic hydrocarbon fraction (fraction A) found by chemical analysis, however, seemed not to be relevant in enabling the bees to distinguish between different waxes. If we choose a lower significance level ($P \leqslant 0.05$), the discrimination between waxes based on fraction A became significant in four of eight cases (Table 6) with a median of 2.68–2.80 errors per bee in the testing phase. Given the median errors of 2.99 and 2.96 in the control experiments, the discrimination between waxes based on fraction A is weak and only significant at a 10-fold lower significance level. There are obviously large differences in the discriminability of the different wax fractions, with fraction D giving the clearest results and fraction A allowing for only a weak (and, according to our strict criterion, statistically insignificant) discrimination.

This work presents new insights into chemical communication in honeybees. For the first time, pure waxes and fractions of waxes with a natural distribution of components were tested using the proboscis extension reflex paradigm, and it is shown that bees use the more polar components (hydroxy alkyl esters, acids and primary alcohols) of the waxes rather than aliphatic hydrocarbons to discriminate between different comb waxes. However, on the basis of our findings, we cannot exclude the possibility that in behavioural contexts other than the proboscis extension reflex honeybees do use hydrocarbons for wax recognition. It is possible that there are wax components that bees can discriminate perfectly well, but such an ability may not be detected with any conditioning paradigm.

Concerning the biological role of comb wax discrimination in honeybees, one may speculate that it could aid in colony recognition and orientation within the dark nest, with the wax combs appearing to the chemoreceptors of the bees as a ‘colourful carpet’. A wild honeybee nest is built and rebuilt over many years and, therefore, different regions of the nest are of different age and can be sensed as different by the bees.

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