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Effect of queen pheromone on worker bees of different ages: behavioural and electrophysiological responses

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Summary — The attraction responses of differently aged worker bees to queen pheromonal blends were studied in a 4-field airflow olfactometer. The effects of the nature of the queen signal – either the synthetic mixture shown to be behaviorally active by Slessor *et al* (1988), or a queen-head extract –, and the rearing conditions were investigated. The composition of the queen-head extract with respect to the constituents of the synthetic blend was chemically analyzed, and differences in amounts of components were discussed. Olfactory-based behavioral responses were elicited by both pheromonal signals; the queen-head extract induced higher responses than the synthetic mixture, the youngest bees of < 5 d old being the most responsive. Similar responses were found in queenless caged bees and in bees reared in a hive, suggesting that attraction to the queen was probably not influenced by prior experience, at least when deprivation to the queen signal occurred after emergence. Electroantennogram responses to the queen signals were recorded concurrently. Olfactory sensitivity was higher to the queen-head extract than to the synthetic pheromone, and was not age-dependent for the age groups tested. No correlation between the maturation of antennal responses and the maturation of behavioral responses was found.

***Apis mellifera ligustica* / queen pheromone / age effect / retinue behavior / olfactory sensitivity / olfactometer**

INTRODUCTION

In the honeybee *Apis mellifera* L, chemicals mediate crucial behaviours such as the search for food sources guided by plant aromas and intraspecific interactions based on pheromones. Among pheromonal signals, queen pheromones appear to play a major role in colony organization. A queen pheromone is known to inhibit the development of worker bee oocytes (Butler, 1954; Pain, 1954), attract males to virgin queens (Gary, 1962), stabilize swarms (Butler, 1960; Simpson and Riedel, 1963), and inhibit queen rearing (Butler, 1954). It also induces the retinue behaviour described as the attraction of a group of worker bees surrounding the queen, an-

tennating, licking and feeding her (Rösch, 1925; Allen, 1955). Although age specialization in tasks among worker bees may be rather flexible according to the amount of brood or food stores, or under stress conditions (Free, 1965; Winston and Fergusson, 1985; Kolmes and Winston, 1988), the retinue behaviour seems to occur at a particular moment in the age polyethism schedule. Workers attending a queen may be of various ages; however, from convergent data it appears that young workers of an age ranging from 1–6 d are mainly involved (Sakagami, 1953; Allen, 1960; Seeley, 1982).

The retinue behaviour has been reproduced under natural conditions in the hive (Gary, 1961; Simpson, 1979) and in cages under laboratory conditions with queen-like signals (Pain, 1961; Pain *et al,* 1962; Pham *et al,* 1982) or Petri dishes (Kaminski *et al,* 1990). Experiments using laboratory tests have shown an age effect in the response of age groups of worker bees to queen extracts, the strongest response occurring for 1–5-d-old bees (Pham *et al,* 1982; Pham-Delègue *et al,* 1991).

The queen signal is a complex chemical blend secreted by several glands such as the mandibular glands (Butler and Simpson, 1958), the Arnhard glands on the tarsi (Lensky and Slabezki, 1981) and the tergal glands (Butler and Simpson, 1965), the mandibular secretion being particularly active in eliciting retinue behaviour (Gary, 1961; Slessor *et al,* 1988). From chemical analyses of the queen secretion it is known that it is a complex blend, made up of a large number of components (Callow *et al,* 1964; Boch *et al,* 1979; Slessor *et al,* 1988), fluctuating according to the individuals (Pain *et al,* 1967), their genetic origin (Crewe, 1982), their physiological stage – virgin or mated – (Crewe and Velthuis, 1980; Slessor *et al,* 1990), and their age (Crewe, 1982; Slessor *et al,* 1990). It has been shown that the pheromonal blend includes a volatile fraction that might partially or completely elicit worker bee responses (Pain, 1961). Recently, a synthetic blend of 5 components has been shown to mimic the activity of the queen blend in eliciting retinue behaviour in worker bees (Slessor *et al,* 1988). These authors showed that this mixture induced activity equivalent to the mandibular gland complex at a level as low as 10^{-7} of that present in a queen.

Most data on the age-dependency of retinue behaviour, obtained either from hive or laboratory experiments, did not allow contact and attraction effects of the queen or queen lures to be differentiated. A bioassay based on olfactory-mediated behaviour using a multichoice olfactometer device adapted to honeybees (Pham-Delègue *et al,* 1990a) showed that worker bees were attracted from a distance by a queen-head extract, and that there was an age-dependency in the attraction effect (Pham-Delègue *et al,* 1991). Based on these data, we attempted to evaluate the efficiency of the synthetic mixture found by Slessor *et al* (1988) in eliciting attraction behaviour. Moreover, we investigated whether an age-effect could be found in the responses to the synthetic mixture as shown for queen-head extract, since Kaminski *et al* (1990) did not succeed in showing such an effect in a pseudo-queen bioassay allowing contact with the synthetic mixture.

These experiments were first conducted with bees reared under cage conditions without being exposed to the queen signal from emergence. It has been reported that during the early days of adult life the olfactory environment might strongly modify the setup and functioning of the olfactory nervous system (Masson and Arnold, 1984; Gascuel and Masson, 1987), as well as behavioural responses to an exposure component such as geraniol (Pham-Delègue *et al,* 1990a). It can be hypothesized that bees reared queenless from

emergence and thus with a reduced experience towards the queen signal would have a different behavioural sensitivity towards that signal compared to bees reared under natural conditions. Moreover, physiological changes may occur in queenless bees that might also affect their behaviour. The development of queenless worker bee oocytes has already been reported (Free, 1987). Laying worker bees appear in queenless hives and the chemical composition of the head extracts of such bees may evolve into a queen-like signal (Crewe and Velthuis, 1980; Crewe and Moritz, 1989). The laying worker bees may behave like false queens, inducing retinue behaviour in the other bees and inhibiting queen rearing (Sakagami, 1958). This therefore led us to investigate whether the maturation of retinue behaviour was affected by the rearing conditions, *ie* prolonged exposure to the queen signal *versus* deprivation from emergence.

Since this study was focused on olfactory-mediated behaviour, it was of interest to concomitantly investigate the antennal detection of the pheromonal signals used in the behavioural assays (queen-head extract, synthetic pheromone). In the honeybee, relatively few data are available on the relationships between behavioural responses and peripheral sensitivity. Although no simple correlate between behaviour and peripheral sensory input would be expected, behaviour must to some extent be mediated by this input (Blaney *et al,* 1986). Vareschi (1971) found that behavioural odor discrimination, based on the conditioned proboscis extension response, could be related to odor classes established from electrophysiological recordings. More recently for some components common with those reported in the work of Vareschi (1971), similar relations between classes of olfactory receptor neurons and behavioural discrimination abilities have been reported by Akers and Getz (1992). Moreover, using combined electroantennogram recordings and conditioned proboscis extension responses, it has been shown that olfactory sensitivity is modified after the learning procedure (De Jong and Pham-Delègue, 1991), suggesting that conditions inducing behavioural modifications may also lead to receptor changes. A maturation process has been established for olfactory sensitivity of general and pheromonal pure components, including queen pheromone constituents, the responses increasing during the first 4 d following emergence, and reaching a plateau by 6–12 d old (Masson and Arnold, 1984; Allan *et al,* 1987). From these data, it could be hypothesized that such an age effect on peripheral sensitivity is a general process likely to be elicited by the queen pheromonal blend. To document this point and to investigate the possible correlates between olfactory sensitivity and behaviour, we recorded antennal responses to both natural and synthetic queen pheromonal blend in individuals of different age groups, following the same conditions as those applied in the behavioural assays.

Thus in this study our aim was to: i) compare the olfactory behavioural responses according to age elicited either by queen-head extract or the synthetic mixture described by Slessor *et al* (1988); ii) evaluate the effect of rearing conditions on behavioural responses; iii) investigate antennal sensitivity towards a queen mandibular signal.

MATERIAL AND METHODS

Queen signal

Two types of queen-like signals were used in the experiments:

– a queen-head extract obtained from *Apis mellifera ligustica* unmated queens. Queen cells

were individually set into cages 2 d prior to emergence. They were reared in groups of 30 workers introduced with an emerging queen at 1 d old, fed with sugar food, pollen and water, and kept in an incubator (33°C, 55%, rh). At the age of 14–17 d, the queens' heads were cut and extracted in dichloromethane (500 µl per head);

– a synthetic blend based on the work of Slessor *et al* (1988, 1990) provided by Phero Tech Inc Company. According to the data of Slessor *et al,* queen-equivalent contained 150 µg (*E*)-9-keto-2-decenoic acid (9ODA), 55 µg (+) and (–) (*R*)-9-hydroxy-2-decenoic acid (9HDA), 13 µg methyl *p*-hydroxybenzoate (HOB), 1.5 µg 4-hydroxy-3-methoxyphenylethanol (HVA), *ie* 220 µg active material.

Chemical analysis

To allow a comparison between the queen stimuli used in the experiments, the constitutive components of the synthetic mixture were dosed in the natural extract. The extract was dried under nitrogen flow. Derivatization was obtained by the addition of 50 µl dichloromethane and a 50-µl mixture of bis-(trimethylsilyl)-trifluoroacetamide (BSTFA) and 1% trimethylchlorosilane (Sigma) left to react for 3 h at room temperature. The sample was then dried under nitrogen flow and solubilized in 100 µl standard solution (0.1 mg hexadecane/ml dichloromethane). The target constituents were quantified by gas chromatography (GC). The gas chromatograph (Carlo-Erba 2000) was complete with an on-column injector, a flame ionization detector, and a HT5 column (SGE, 25 m length, 0.32 mm internal diameter, 0.1 µm film thickness). The carrier gas was hydrogen. The temperature program was 40°C to 100°C at 10°C/min, 100°C to 150°C at 3°C/min, and 150°C to 300°C at 10°C/min. Three injections of the sample were given. The response coefficients of the pheromonal constituents were obtained by injecting reference components (0.01 µg to 1 µg of 9ODA, 9 HDA, HOB, HVA per injection after prior derivatization).

The identification of the components was conducted using coupled gas chromatography–mass spectrometry, by comparing the spectra of the components found in the extract to those of the reference components. GC was conducted on a Varian 3400 (split/splitless injector) set with an HT5 column (SGE, 25 m length, 0.22 mm internal diameter, 0.1 mm film thickness). The gas carrier was helium at 1 ml/min. The temperature program was 50°C to 100°C at 10°C/min, 100°C to 150°C at 3°C/min, and 150°C to 300°C at 10°C/min. The mass spectra were obtained by electron impact using a INCOS 50 (Finnigan MAT) coupled to the GC (source pressure 0.015 Torr, 70 eV).

Biological material

Combs were collected from outside hives in June and July and stored in an incubator (33°C). To control age homogeneity, *Apis mellifera ligustica* L worker bees < 4 h old were randomly collected and divided into 2 samples. One was labelled with a color spot on the thorax and reintroduced into a rearing hive with a 1-yr-old mated queen and ≈ 10 000 workers. The other was reared queenless in cages of ≈ 50 individuals of the same age, fed with sugar and pollen food, provided with water, and kept in an incubator (33 °C, 55% rh).

Two behavioural assays were conducted:

– responses to either the natural extract or the synthetic mixture were tested on individuals of the queenless groups at every age between 1 and 7 d;

– to evaluate the influence of the rearing conditions at the ages required for the experiments, *ie* 2, 4, 8, 15 and 21 d, labelled bees were removed from the rearing hive to be tested on the same day as the caged bees of the same age.

Antennal responses were recorded from 2, 4, 8, 15 and 21-d-old worker bees reared under hive conditions.

Behavioral assay

Experimental device

A 4-field airflow olfactometer previously set for aphids (Petterson, 1970) and for parasitoid insects (Vet *et al,* 1983) and adapted to honeybees (Pham-Delègue, 1990a) was used. The device was made of transparent perspex with a 4-arm star-shape observation chamber (35.2 cm width, 2 cm height). A central suction created 4

contiguous flow fields (0.25 l/h at the entrance to each arm). Each airflow passed through a glass vial containing a piece of filter paper soaked either with the queen pheromone solution (1 test field) or the solvent (3 control fields). The device was set on a table diffusing red light (700 lux). The observations were conducted on a monitor screen (Sony Trinitron) connected to a camera (Nikon TM-560). The experiments were carried out in a climatized dark room (24°C ± 1°C, 70% rh).

Protocol

Bees were individually introduced into the center of the observation chamber, and their positions recorded over 5-min observation periods, after which the insects were discarded. The bees were given a choice between 1 odor field *versus* 3 unscented fields, which provided a 0.25 probability of visiting the odor field. In the odor field was diffused either 1-queen equivalent of the natural pheromonal extract or 1.5 queen-equivalent of the synthetic pheromone. From preliminary experiments, it appeared that the odor stimulus remained active for 4 tested bees when using the extract, whereas the activity of the synthetic blend lasted only for 1 bee. Therefore, the lures were renewed every 4 tested bees with the extract, and every bee tested with the synthetic pheromone. The location of the scented field was rotated after every bee tested to make sure that responses were cued to the olfactory signal, and the observation chamber was cleaned with ethanol every 4 tested bees to prevent the deposition of marking scents.

To measure the response to the pheromonal simulus, the time spent in the odor field was recorded over the 5-min observation period for each bee and then averaged over groups of 8 bees.

Two pairs of experimental groups were used:

– one pair testing the effect of the queen stimulus (natural *versus* synthetic) with individuals reared queenless from emergence in cage conditions and tested either with the natural or the synthetic queen signal;

– one pair testing the effect of the rearing conditions, with individuals reared either queenless in cages or under hive conditions and then tested with the natural extract. Individuals in the paired experimental groups were tested alternately on the same day and at the same age (for age groups; see *Biological material).*

Statistics

Within each age group and each experiment to evaluate the attraction effect of the stimuli the proportion of individuals (out of 8 tested) spending > 25% time in the odor field (*ie* > 75 s) was tested by a χ^2 test (1 df).

Individual time values (in s) recorded from the odor field during the observation period were then analyzed. The normality of data distribution within each pair of experiments was first checked using a Kolmogorov–Smirnov test. In each pair of experimental groups, age effect and either stimulus or rearing conditions effect was analyzed using a multifactor analysis of variance followed by a least significant difference (LSD) test applied to the age effect.

Electrophysiological recordings

Stimuli

One queen-equivalent of the queen-head extract (QE) and 10 queen-equivalents (according to Slessor *et al,* 1988, 1990) of the synthetic pheromone (SP) were used as stimuli. The stimuli were prepared by drying under nitrogen flow solutions containing the required amount of pheromonal material and then diluting the dry material in 20 µl paraffin oil at 35°C; 1 20-µl mixture of paraffin oil and 1-hexanol (10^{-3} v/v) was used as reference (HE); 20 µl pure paraffin oil was used as blank stimulation (BL). Each solution (20 µl) was deposited on a piece of filter paper set into a glass Pasteur pipet.

Recordings

A constant flow (60 cm/s) of purified and humidified air passed through a 7-mm id glass tube. The thin tip of the Pasteur pipet containing the odor source was placed in a hole placed 8 cm from the outflow extremity of the glass tube. Stimulation was delivered by a 40 cm/s flow passing through the Pasteur pipet for 1 s. A switch gate ensured a resultant outflow of 60 cm/s. Electrodes were filled with Ringer solution

and connected to a high-impedance DC amplifier. Electroantennograms were recorded on an oscilloscope screen on which the amplitude values were measured. The precision of the measurements was ± 0.025 mV.

Protocol

The experiments were conducted for 5 consecutive d in June. Three individuals in every age group (2, 4, 8, 15 and 21 d old) collected from the experimental hive were tested daily. In each age group, the responses of 15 individuals were recorded. The recordings were obtained from isolated heads set in the outflow 5 mm from the stimulating device output at 25°C. The stimulating sequence was the following: HE_1, BL_1, QE, SP, BL_2, HE_2. This sequence and the reverse sequence were used alternately from one bee to the next. The inter-stimulation period lasted for 30 s.

Statistics

The normal distribution of the 75 values (5 ages x 15 individuals) of the stimulation groups BL_1, BL_2, HE_1, HE_2 was checked using a Kolmogorov–Smirnov test. Within each of these stimulation groups, 1-way analysis of variance (ANOVA) was used to analyze the age effect.

For stimulation groups QE and SP, statistical evaluation was performed on electroantennogram values modified according to Renou *et al* (1988), to take into account the responses to the blank and the reference stimuli:

$$E_{if} = E_{im} - E_{bm}$$

$$E_{im} = (2 \times E_i \times HE_1) / (HE_1 + HE_2)$$

E_{if}: final value for stimulus *i*, used for statistics; E_{im} : value for stimulus *i* modified according to the response to the reference stimulus HE; E_{bm}: value for the blank stimulus modified according to the response to the reference stimulus HE; E_i: observed value for stimulus i; HE_1: observed value for the reference stimulus HE tested closer to stimulus *i* in the sequence; HE_2: observed value for the reference stimulus HE tested more distant in time from stimulus *i*. The normal distribution of the modified values within the stimulation groups QE and Sp was checked using a Kolmogorov–Smirnov test. Within each stimulation group, the age effect was analyzed by ANOVA. Between the 2 stimulation groups, comparison of the responses was made using a *t*-test on 2 paired samples.

RESULTS AND DISCUSSION

Chemical analysis

The chemical analysis of the natural extract found 1 queen-equivalent to be 312 ± 90 µg 9ODA and 18 ± 2 µg 9HDA. The other components, HOB and HVA, found to be active by Slessor *et al* (1988) could not be detected in the extract even after scanning for the characteristic ions of their trimethylsilyl derivatives (HOB: 135, 209, 224; HVA: 179, 209, 312).

For 9ODA and 9HDA, our data are included in the range of values reported in the bibliography for 15–20-d unmated queens. The amounts may fluctuate from 10–1 000 µg for 9ODA and from 5–280 µg for 9HDA (Pain *et al,* 1974; Crewe, 1982; Crewe and Moritz, 1989; Slessor *et al,* 1990).

The lack of HVA in our extract obtained from unmated queen heads is not surprising since this component has been detected in mated queens only, up to 2 µg per queen (Crewe and Velthuis, 1980; Slessor *et al,* 1990) except in *Apis mellifera intermissa* where HVA is secreted by both unmated and mated queens as well as by worker bees (Crewe and Moritz, 1989). Amounts of HOB have also been reported to be much higher in mature laying queen than in virgin queens (16 µg *versus* 0.4 µg) (Slessor *et al,* 1990).

Compared to the data of Slessor *et al* (1988, 1990), it appeared that the amount of 9ODA in our extract was about twice that in the synthetic pheromone (150 µg), whereas the amount of 9HDA detected in the extract was ≈ 3-fold lower than that reported by Slessor *et al* (55 µg). The differences in the amounts of 9ODA and of

9HDA as well as the absence of HOB and HVA in our extract make the comparison between the 2 types of queen-like signals rather difficult. However, if we refer to the amount of the main component, 9ODA, it can be considered that the natural extract was ≈ 2-fold more concentrated than the synthetic blend. The inter-stimulus differences in the behavioural and electrophysiological responses will be discussed on this basis.

The semiochemical content of the queen mandibular extract (unmated 14–17-d-old queens) is consistent with data obtained from queens of similar physiological status, as reported by Slessor *et al* (1990) in unmated 12-d-old queens (205 µg 9ODA, 29 µg 9HDA, 0.4 µg HOB, and no HVA).

Behavioral assay

Effect of the nature of the queen signal (table I)

When considering the number of individuals spending > 25% of the observation duration in the odor field, a significant attraction ($P < 0.05$) to the queen extract was found in all age-groups except the 7-d-old group, whilst the synthetic pheromone elicited a significant attraction in all age-groups except the 1-d- and the 6-d-old groups.

Kolmogorov–Smirnov test applied to the time spent in the odor field led to DN values that were consistently below 0.13 (DN = $1.36/\sqrt{n}$, with n = 2 stimuli x 7 age-groups x 8 individuals), indicating that the responses followed a normal distribution, thus allowing the use of parametric statistical tests.

Multifactor analysis of variance (MANOVA) showed significant differences on both the stimulus effect (F = 9.8; 1,98 df; $P <$ 0.01) and the age effect (F = 5.2; 6,98 df; $P < 0.001$) without interaction between the 2 effects; 1 to 4-d-old age groups elicited significantly higher responses than the older bees (LSD test).

From these data, it appeared that: i) olfactory-based behavioural responses could be elicited by both pheromonal signals; ii) an age effect was found, the youngest bees of < 5 d-old being the most responsive; iii) the queen extract induced stronger responses than the synthetic pheromone under our experimental conditions.

Table I. Mean time spent (± SD) over a 300-s observation period, in the odor field, according to age (8 bees tested per age group) and stimulus (QE: queen-head extract; SP: synthetic pheromone).

	Age (d)							*Stimulus effect*
	1	*2*	*3*	*4*	*5*	*6*	*7*	
QE	200 (40)	175 (46)	183 (88)	172 (95)	95 (20)	111 (59)	88 (23)	F = 9.8; df: 1,98
SP	96 (56)	140 (64)	135 (83)	144 (83)	103 (72)	66 (22)	87 (23)	$P < 0.01$
Age effect	a	a	a	a	b	b	b	
	F = 5.2; df: 6,98; P = 0.001							

Stimulus and age effects were analyzed using a multifactor analysis of variance, followed for the age effect by a least significant difference test for multiple range analysis (letter indexation: responses without common letters are significantly different at $P < 0.05$).

The lower effectiveness of the synthetic mixture was related to lower amounts of components – 2-fold less 9ODA – and to the reduced number of constitutive components compared to the queen extract.

The age effect found in the olfactory responses to the pheromonal signals confirms previous data obtained from a larger range of age groups with caged queenless worker bees tested in the olfactometer device for queen-head extract (Pham-Delègue *et al,* 1991). It is also consistent with the age effect reported for experimental bees reared under the same conditions in the present study and stimulated with the same kind of queen-head extract, but tested in a bioassay allowing contact with the queen lure (Pham *et al,* 1982). This suggests that the age dependency in the response of worker bees to the queen signal may partially be an olfactory-based response. However, these results are in contradiction with those of Kaminski *et al* (1990), which did not show an age effect in a bioassay allowing contact with lures prepared with the synthetic mixture. This is probably not related to the synthetic stimulus used, since our experiments showed that attraction response could be elicited by both a head extract and the synthetic mixture, although the queen extract was more effective. It may rather rely on the fact that in the bioassay designed by these authors, groups of mixed-age worker bees were tested to be closer to the hive situation, whereas in previous experiments showing an age effect, a similar bioassay was used but with groups of bees of exactly the same age (Pham *et al,* 1982). Since it appeared from experiments on recruiting behaviour that bees of the same age class seemed to interact together more than with bees of other age classes (Pham-Delègue *et al,* 1990b), it can be hypothesized that inter-individual relations may differ markedly according to the age of the individuals interacting. Therefore, we may assume that an attraction effect would appear more clearly in homogeneous age groups rather than in mixed age groups. This kind of interaction between individuals of the same age may also occur within the hive, where it is known that bees of similar ages are involved in retinue behaviour (Seeley, 1982). Thus, testing homogeneous age groups to determine an age effect in the attraction response to the queen signal would be an appropriate procedure.

The age effect in the attraction response to queen signals although consistent with data reported from hive observations (Sakagami, 1953; Allen, 1960; Seeley, 1982) remains questionable, since queenless worker bees were used to perform the test. Testing queenless caged bees may lead to work on particular individuals, especially in the oldest bees with modified biochemical and pheromonal properties and with different experience towards the queen signal, which would affect their behavioural sensitivity to queen signals. This is why we designed an experiment to compare the behavioural responses of caged queenless bees to those of hive bees.

Effect of rearing conditions (table II)

Here, 5 age-groups spread over the lifespan of the worker bees set up with bees either from a rearing hive or maintained queenless in cages were tested for their reaction to the queen-head extract.

When considering the number of individuals spending > 25% of the observation duration in the odor field, a significant attraction ($P < 0.05$) to the queen extract was found in all age groups for hive bees, whilst a significant attraction appeared only at 4 d in the caged bees.

Kolmogorov–Smirnov test applied to the times spent in the odor field led to DN val-

Table II. Mean time spent (± SD), over a 300-s observation period in the odor field according to age (8 bees tested per age group) and rearing conditions.

	Age (d)					*Rearing effect*
	2	*4*	*8*	*15*	*21*	
Hive	145 (83)	194 (69)	163 (89)	125 (47)	103 (31)	$F = 3$; df : 1,70
Cage	167 (108)	132 (80)	134 (94)	86 (42)	71 (20)	NS
Age effect	ab	a	ab	bc	c	
		$F = 3.5$; df : 4.70; $P < 0.05$				

Rearing and age effects were analyzed using a multifactor analysis of variance, followed for the age effect by a least significant difference test for multiple range analysis (letter indexation: responses without common letters are significantly different at $P < 0.05$).

ues that were consistently < 0.15 (DN = $1.36/\sqrt{n}$, with $n = 2$ rearing conditions x 5 age-groups x 8 individuals), indicating that the responses followed a normal distribution, thus allowing the use of parametric statistics.

The analysis of time spent in the odor field showed a significant age effect ($F = 3.5$, 4,70 df; $P < 0.05$). The highest response was observed in the 4-d-old bees, which was significantly higher than that in the 15- and 21-d groups, but not the 2- and 8-d-old groups. The rearing conditions effect was not significant. No interaction between the 2 effects was found.

This set of experiments: i) confirmed the occurrence of an age effect in the attraction response to a queen extract, the 4-d-old bees being the most responsive; ii) showed no significant difference in the responses according to the rearing conditions.

The age effect found in these experiments was not as strong as that reported in the first set of experiments, probably due to the reduced number of age groups tested and the choice made in the ages tested. Thus the main change in the intensity of the attraction response to a queen lure seems to occur after 4 d old. In the present study, 3 out of 5 ages tested were above this age, resulting in an averaging of the responses. However, it consistently appeared that the 4-d-group was the most responsive.

The comparison of the responses according to the rearing conditions did not show significant differences. However, it can be noticed that the responses of the hive bees tended to be higher than those of the caged bees. Deprivation of the queen signal from emergence to the ages tested did not induce a significant change in the normal maturation of the attraction response to the queen signal. This may seem somewhat surprising if we refer to morphofunctional data establishing that the olfactory experience (olfactory deprivation) applied during a critical period lasting from 3 d before emergence to 4–8 d of adult life induced changes in synaptic density of the antennal lobe (Gascuel and Masson, 1987), as well as in olfactory sensitivity (Masson and Arnold, 1984). Moreover, olfactory exposure to a component such as geraniol was shown to induce changes in olfactory-mediated behaviour (Pham-

Delègue *et al,* 1990a). The fact that no drastic changes occurred in the maturation of the responses to the queen signal although bees were deprived of this signal from emergence suggests that there would be little flexibility in responses to a signal with strong biological meaning. It may also be assumed that the period when exposure/deprivation to this kind of signal would induce behavioural changes has to cover pre-imaginal stages. Further experiments are currently in progress to investigate these points.

The similar response profiles exhibited by both hive and caged bees show that responses of bees reared under artificial conditions are valuable in representing natural responses, at least as regards responses to the queen signal.

Electrophysiological recordings (table III)

Electroantennogram responses to 1-hexanol (HE_1 and HE_2) were higher than the antennal responses to the queen signals (QE and SP) which were, however, higher than those obtained with the unscented control (BL_1 and BL_2). Kolmogorov–Smirnov test applied to the electroantennogram responses to the blanks and the 1-hexanol led to DN values that were consistently < 0.16 ($DN = 1.36/\sqrt{n}$, with $n = 5$ age groups x 15 individuals), indicating that the responses followed a normal distribution thus allowing the use of parametric statistics. For both the reference stimulus and the blank stimulus no significant age effect was found.

For responses gained from stimulation groups QE and SP, normal distribution of modified data was shown (DN < 0.16). Significant differences according to age were found neither in responses to QE nor to SP. From the modified data, it appeared that the queen extract elicited significant higher electroantennogram responses than the synthetic pheromone ($P < 0.001$).

Therefore, the olfactory sensitivity of the honeybees appeared: i) to be higher in response to the queen-head extract than to the synthetic mixture; ii) not to be age-dependent, at least for the age groups tested.

One queen equivalent of the natural extract induced higher electroantennogram response than 10 queen-equivalents of the synthetic pheromone. Referring to the

Table III. Electroantennogram responses in mV (± SD) according to age (15 bees tested per age group) and stimulus.

Stimulus	*Age (d)*				
	2	*4*	*8*	*15*	*21*
BL1	0.40 (0.13)	0.38 (0.10)	0.45 (0.12)	0.37 (0.13)	0.48 (0.13)
BL2	0.42 (0.18)	0.38 (0.11)	0.43 (0.10)	0.37 (0.15)	0.50 (0.17)
HE1	1.99 (0.75)	2.03 (0.56)	2.17 (0.72)	1.88 (0.56)	2.37 (0.76)
HE2	1.94 (0.74)	1.98 (0.60)	2.21 (0.62)	1.77 (0.54)	2.25 (0.75)
QE	0.65 (0.20)	0.70 (0.19)	0.79 (0.18)	0.62 (0.19)	0.77 (0.28)
SP	0.60 (0.18)	0.58 (0.15)	0.71 (0.19)	0.57 (0.16)	0.71 (0.17)

BL1 and BL2: blanks; HE1 and HE2: 1-hexanol; QE: queen-head extract; SP: synthetic pheromone.

chemical composition of the queen extract, the stimulation using the synthetic pheromone was ≈ 5-fold more concentrated for the 9ODA and 30-fold for the 9HDA. The higher responses elicited by the natural extract might be due to the fact that other constitutive components of those common to the synthetic blend had been detected by the bees.

Although the age effect was not significant among the age groups tested, it could be observed that for all stimuli the electroantennogram responses were higher at 8 and 21 d old. Masson and Arnold (1984) and Allan *et al* (1987) reported that olfactory maturation took place during the first week after emergence and that sensitivity reached a maximum by 6–12 d. In both cited works, the same olfactory maturation process was obtained for a range of pure odorants, including pheromonal compounds such as 9ODA and 9HDA. Such a maturation process could not be found from our data, probably due to the fact that we did not test as many age groups as the mentioned authors.

When referring to the maturation process observed in the behavioural responses to the queen signal, showing a maximum attraction to the stimulus at 4 d, no direct correlates between antennal sensitivity and behavioural responses could be drawn. It seemed that whereas the maturation of the olfactory antennal system was a general process occurring for general or specific odors, the maturation of behavioural responses was specific to queen pheromonal components. Thus it seems that bees < 4 d old performing retinue behaviour would be less olfactory-sensitive to queen pheromone components than older bees of 8 d likely to be involved in other tasks. The ability to detect an odor at the antennal level does not necessarily involve a behavioural response, as also underlined by Allan *et al* (1987), who found electroantennogram responses to some sting-gland components that were ineffective in inducing alarm behavioural responses as reported by Collins and Blum (1982). The regulation mechanisms responsible for antennal and behavioural maturation processes appear to be different, since it has been shown that topical application of a juvenile hormone analogue reduced the behavioural sensitivity to alarm pheromones without affecting the electroantennogram response (Robinson, 1987). This author suggested that the hormonal effects inducing behavioural changes may occur in the central nervous system and not at the level of antennal receptors.

In conclusion, this study indicates a maturation process in olfactory-mediated behavioural responses of worker bees regarding both a queen extract and a synthetic mixture shown to be active in eliciting retinue behaviour. The changes with age in behavioural responses are consistent with the polyethism of tasks within the hive, the bees of < 6 d being more likely to attend the queen (Sakagami, 1953; Allen, 1960; Seeley, 1982), suggesting that this specialization in task may be at least partially mediated by olfactory cues. The behavioural responses mediated by the queen pheromone did not correlate with antennal sensitivity, and seemed to be rather weakly flexible according to the experience towards the queen signal and to the colony environment, at least when applied after emergence.

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Résumé — Effet de la phéromone royale sur des ouvrières d'abeilles de différents âges : réponses comportementales et électrophysiologiques. Dans la colonie d'abeilles, la phéromone royale induit, principalement chez les ouvrières âgées de moins de 6 j, un comportement de cour, décrit comme l'attraction d'un groupe d'ouvrières entourant la reine, effectuant des contacts antennaires, la léchant et la nourrissant. Dans ce travail, nous avons étudié le comportement d'attraction d'ouvrières d'abeilles (*Apis mellifera ligustica* L), appartenant à différentes classes d'âges, vis-à-vis d'un signal de reine. Ce comportement a été observé dans un olfactomètre dynamique à quatre voies, en fonction de la nature du signal royal et des conditions d'élevage des ouvrières. Le signal proposé a consisté soit en un mélange synthétique démontré comme étant attractif pour des ouvrières (Slessor *et al,* 1988), soit en un extrait de têtes de reines *Apis mellifera ligustica* vierges âgées de 14-17 j. Les composés communs aux 2 types de signaux ont été dosés et identifiés dans l'extrait par des techniques de chromatographie en phase gazeuse (CPG) et de CPG couplée à la spectrométrie de masse. Des valeurs de 312 ± 30 µg 9ODA et 18 ± 2 µg 9HDA ont été trouvées dans l'extrait; par contre, le HOB et le HVA n'ont pu être détectés. Ces quantités diffèrent de celle du mélange synthétique (150 µg 9ODA, 55 µg 9HDA, 13 µg HOB, 1,5 µg HVA), mais sont proches de celles rapportées dans la littérature pour des reines vierges de 2 semaines. Malgré ces différences de composition, les 2 types de signaux se sont avérés attractifs vis-à-vis des ouvrières, les abeilles de moins de 5 j étant les plus fortement attirées (tableau I). La comparaison des réponses comportementales d'ouvrières privées de reine à l'émergence et élevées par groupes d'âge homogènes en cagettes, et d'ouvrières maintenues en colonie, ne montre pas de différence significative (tableau II). L'ensemble de ces résultats montre que les réponses comportementales des ouvrières vis-à-vis de la phéromone de reine sont : i) dépendantes de l'âge des ouvrières, ii) supérieures pour un extrait de tête par rapport au mélange synthétique utilisée, iii) peu sensibles à l'expérience des ouvrières vis-à-vis de ce signal après l'émergence, iv) cohérentes avec les données de la littérature se rapportant au comportement de cour observé en conditions naturelles. Le comportement observé en olfactomètre étant basé sur une réponse olfactive, la sensibilité antennaire des ouvrières aux signaux phéromonaux a été étudiée parallèlement par l'enregistrement d'électro-antennogrammes (tableau III). Il apparaît que (i) les réponses antennaires sont supérieures pour l'extrait par rapport à la phéromone de synthèse, (ii) la sensibilité olfactive est indépendante de l'âge, pour les groupes d'âge étudiés, (iii) la maturation des réponses antennaires n'est pas corrélée à celle des réponses comportementales vis-à-vis de la phéromone royale. Le travail présenté montre la validité de l'essai comportemental utilisé pour mettre en évidence des phénomènes de maturation des réponses comportementales d'ouvrières d'abeilles vis-à-vis de la phéromone royale, et souligne que ces réponses ne sont pas strictement sous la dépendance de l'état de maturation du système nerveux olfactif périphérique.

***Apis mellifera ligustica* / phéromone royale / effet de l'âge / comportement de cour / sensibilité olfactive / olfactomètre**

Zusammenfassung — Wirkung des Königinpheromons auf Arbeiterinnen verschiedenen Alters: Verhaltens- und elektrophysiologische Reaktionen. Im Bienenvolk löst das Königinpheromon besonders bei Arbeitsbienen im Alter von

mindestens 6 Tagen, das Verhalten der Hofstaatbildung aus, das sich folgendermaßen beschreiben läßt: Anlockung einer Gruppe von Arbeiterinnen rund um die Königin, die Kontakte mit den Fühlern herstellen und die Königin belecken und füttern. In dieser Arbeit haben wir das Verhalten der angelockten Arbeiterinnen von *Apis mellifera ligustica* verschiedener Altersklassen gegenüber einem Signal der Königin studiert. Dieses Verhalten wurde in einem dynamischen 4-Weg-Olfaktometer hinsichtlich der Art des Signals der Königin und der Aufzuchtbedingungen der Arbeiterinnen beobachtet. Dieses Signal bestand entweder aus einer synthetischen Mischung, die sich gegenüber Arbeiterinnen als attraktiv erwiesen hat (Slessor *et al,* 1988) oder aus Kopfextrakten von unbegatteten Königinnen mit einem Alter von 14 Tagen von *A mellifera ligustica.* Die für beide Typen des Signals gemeinsamen Komponenten wurden dosiert und in den Extrakten durch Gaschromatographie (GCMS) und durch GC gekoppelt mit einem Massenspektrometer bestimmt.

In den Extrakten wurden Werte von 312 ± 30 µg 9ODA und 18 ± 2 µg 9HDA gefunden; HOB und HVA hingegen wurden nicht entdeckt. Diese Mengen sind von denen in der synthetischen Mischung verschieden (150 mg 9ODA, 55 µg OHDA, 13 µg HOB, 15 µg HVA), aber sie sind ähnlich wie die in der Literatur für unbegattete, 2 Wochen alte Königinnen beschriebenen. Ungeachtet dieser Unterschiede in der Zusammensetzung sind beide Typen von Signalen zweifellos für Arbeiterinnen attraktiv, wobei Bienen in einem Alter von weniger als 5 Tagen am stärksten angelockt werden (Tabelle I). Ein Vergleich der Verhaltensreaktionen von seit dem Schlupf weisellosen und in gleichaltrigen Gruppen gekäfigten Arbeiterinnen und von Bienen aus Völkern ergab keine signifikanten Unterschiede (Tabelle II).

Eine Zusammenfassung der Versuche zeigt, daß die Verhaltensreaktionen der Arbeiterinnen gegenüber dem Königinpheromon i) vom Alter der Bienen abhängen, ii) für Kopfextrakte stärker sind als für das benutzte synthetische Gemisch, iii) für die Erfahrung der Arbeiterinnen gegenüber dem Signal nach dem Schlüpfen wenig empfindlich sind, iv) mit den veröffentlichten Resultaten über das Verhalten der Bienen des Hofstaates im natürlichen Volk übereinstimmen.

Das Verhalten im Olfaktometer basiert auf Geruchsreaktionen, deshalb wurde die Antennensensibilität der Arbeiterinnen auf Pheromonsignale gleichzeitig mit der Registrierung von Elektroantennogrammen studiert (Tabelle III). Es zeigte sich, daß :

– die Antennenreaktion gegenüber dem Extrakt stärker ist als gegenüber dem synthetischen Pheromon,

– die Geruchsempfindlichkeit bei den untersuchten Altersgruppen vom Alter unabhängig ist, und

– die Reifung der Fühlerreaktionen mit der Reifung der Verhaltensreaktionen gegenüber dem Königinpheromon nicht korreliert ist. Diese Arbeit zeigt die Brauchbarkeit von Verhaltensversuchen wie sie zum Nachweis des Phänomens der Reifung von Verhaltensreaktion der Arbeitsbienen gegenüber dem Königinpheromon benutzt wurden, und unterstreicht, daß diese Reaktionen nicht strikt vom Reifezustand des peripheren olfaktiven Nervensystems abhängen.

***Apis mellifera ligustica* / Königinpheromon / Alterseinfluß / Hofstaatbildung / Geruchsempfindlichkeit / Olfaktometer**

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