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Mechanisms of food provisioning of honeybee larvae by worker bees

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SUMMARY

Although it has clearly been demonstrated in previous studies that honeybees inspect their worker brood in a non-random fashion, it is still unclear which signals and cues worker bees use to monitor the nutritional state of their brood. Here we show that worker bees can recognize and quantify the larval food present in a brood cell olfactorily and identify potential mechanical signals produced by the brood. There is no evidence for additional chemical hunger signals produced by the larvae. However, the pattern of movement of larvae within their cells changes with their nutritional state and might provide additional information to nurse bees.

Key words: *Apis mellifera*, behaviour, communication.

INTRODUCTION

Worker honeybees (*Apis mellifera* L.) provide their larvae in the brood cells with a larval food which varies in quality and quantity between castes, sexes (Patel et al., 1960; Matsuko et al., 1973; Brouwers et al., 1987) and larval instars (Jung-Hoffmann, 1966; Crailsheim, 1992). Experimentally food-deprived larvae are inspected more frequently than untreated controls, indicating that there are signals or cues providing the nurse bees with information about the nutritional state of the larvae (Huang and Otis, 1991a). Chemical signals produced by the eggs and brood of honeybees are known to be used by worker bees in a variety of contexts (Koeniger and Veith, 1983; Ratnieks and Visscher, 1989; Le Conte et al., 1990; Trouiller et al., 1991; Huang and Otis, 1991b; Le Conte, 1994; Le Conte et al., 1995; Châline et al., 2005). Cuticular hydrocarbon patterns of honeybee larvae provide information about the age and caste of the larva (Aumeier et al., 2002). Worker bees can olfactorily distinguish between the odours of male and female larvae (Sasaki et al., 2004). However, it is still entirely unclear how worker bees gain information about the nutritional state of larvae.

In addition to chemical signals, mechanical signals and cues play a significant role in social insect communication systems (Kirchner, 1997). Mechanical hunger signals have been described in several wasp species (Ishay and Ikan, 1968; Ishay and Landau, 1972; Ishay and Brown, 1975). The aim of the present study was therefore to identify chemical as well as mechanical signals and cues produced by larvae and larval food that can potentially provide worker honeybees with information about the food supplies of the larvae, and to clarify whether worker bees can perceive these signals.

MATERIALS AND METHODS

All observations and experiments were performed using queenright colonies of *Apis mellifera carnica* L. kept on campus at Ruhr-University, Bochum, Germany.

Observations of feeding behaviour were performed in a small colony unit of 2000–3000 bees using combs, which allowed us to videotape single brood cells longitudinally attached to Perspex sheets, using an infrared-sensitive video camera. This method permitted the unambiguous identification of ‘feeding visits’ by monitoring the disposal of food drops by worker bees. As already assumed by Huang and Otis (Huang and Otis, 1991a), feeding visits take at least 10 s. Moreover, worker bees bend deeply into the cell and remain nearly motionless in this position during feeding. In contrast, a worker bee engaging in cell inspection frequently shifts during the cell visit or even adjourns the inspection after a few seconds. This knowledge was applied to detect feeding visits without direct insight into brood cells in a second experiment, in which a regular two-frame observation hive was used to videotape larger areas of uncapped brood.

Food deprivation was ensured either by preventing the worker bees from accessing the cells by using mesh screening or by inserting wooden sticks (2 mm diameter) through the Perspex front screen of the observation hive into single cells. The latter method allowed the workers to patrol on the rims of the cells but not to inspect or feed the larva inside. In both experiments each larva (fifth instar) was observed for 30 min subsequent to food deprivation.

For classical conditioning of worker bees (always taken from the same hive), the proboscis extension reflex (PER) training was employed as described by Bitterman and colleagues (Bitterman et al., 1983) using pentane extracts of food-deprived or control larvae (60 larval equivalents each; fifth instar; 4.5 h or no food deprivation, respectively) as well as pentane extracts of larval food. For extraction, larvae or larval food was kept in n-pentane (Uvasol grade; Merck, Darmstadt, Germany) for 1 h. The concentrated extracts were supplied on filter paper in glass pipettes. In order to simulate the volatility of the compounds in the bee hive the pipettes were kept at 35°C. Bees were trained to the conditioned stimulus (CS) in three series (in the case of poor training success, one or two extra conditioning series were performed). Then the CS and the reference scent (RS) were offered alternately (usually eight times). PERs to the CS were rewarded. In addition, reactions to pure solvent were checked twice.

The spontaneous choice behaviour of worker bees was tested using a slightly modified version of the quadruple choice assay described by Rosenkranz (Rosenkranz, 1993). Single bees were observed for 4 min under dim red light at 35°C in an experimental chamber in which four wells in the ground provided larval food in varied amounts (two cells always contained the same sample). A mesh screen prevented direct access to the food samples, ensuring that the choice behaviour of the bees was exclusively driven by differences perceived olfactorily.

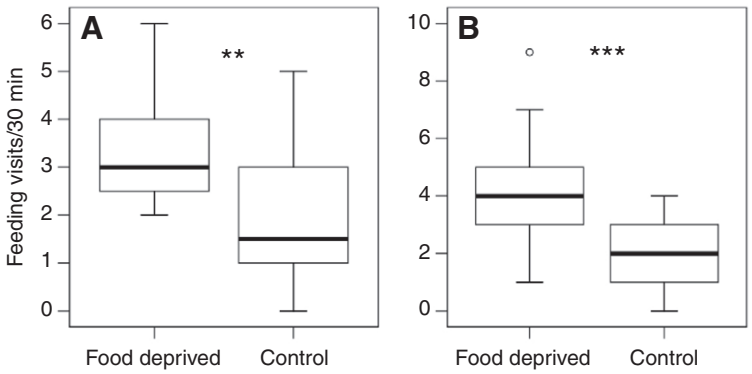

Fig. 1. (A) Number of food provisioning visits to brood cells containing food-deprived (N=15) and control larvae (N=26) per 30 min. Food-deprived larvae (2 h food deprivation) are fed significantly more often than the controls (**P<0.01; U-test). (B) The same effect is found when workers are allowed to patrol on the rims of the cells during food deprivation (3 h food deprivation) of the larvae (N=54 each, ***P<0.001; Mann–Whitney U-test, outlier indicated by open circle).

Pentane extracts of food-deprived and control larvae concentrated to 1 larval equivalent per microlitre were analysed in a Hewlett-Packard 5890 II gas chromatograph equipped with a FID-detector and HP 3365 Series II Chemstation. The analyses were performed with a non-polar fused silica column (DB-5, 30 m×0.32 mm i.d.×24 μm), which was operated with a standard 4-step temperature program.

Possible mechanical cues to the nutritional state of the larvae were recorded by measuring the velocity of larval rotation after varying periods of starvation. For this experiment larvae from eight colonies were used.

RESULTS

Worker bee larvae that were experimentally food deprived for 3 h were fed significantly more frequently afterwards (Fig. 1A). This effect can obviously not be explained by changes in the smell of the comb, which was inaccessible for the bees during food deprivation, because the very same effect was found when the cell rims were accessible for the bees while the content of the brood cells was inaccessible (Fig. 1B).

In the laboratory, groups of worker bees were trained to exhibit a PER in response to the odour of larvae. They can smell larval odour extracts and respond with rates of PER reactions as high as with floral odours. Bees trained to respond to the smell of well-fed control larvae as the CS but not to the smell of larvae that had been food deprived discriminated significantly (Fig. 2). Bees that had been trained to respond to the smell of hungry larvae, however, learned as well, but were not able to discriminate. As this result indicates that the difference between the two odours is quantitative rather than qualitative, bees were trained to respond to the smell of larval food. Again, they learned to respond to this odour well (Fig. 3A), and they discriminated between different concentrations significantly when they had been trained to higher concentrations, but not after training to lower ones (Fig. 3B).

In order to test whether worker bees can spontaneously discriminate between different quantities of larval food, single worker bees were

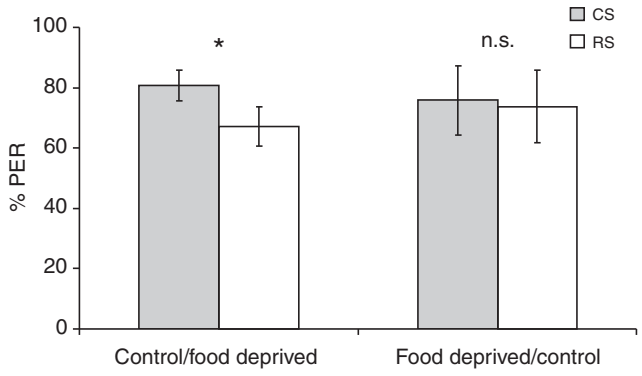

Fig. 2. Bees can be trained to exhibit a proboscis extension reflex (PER) in response to the odour of larvae. They can discriminate between the smell of well-fed control larvae used as the conditioned stimulus (CS) and the smell of larvae that have been food deprived (reference scent, RS; N=76 bees, *P<0.05; Wilcoxon test). Bees trained to respond to the smell of hungry larvae respond to the smell of well-fed controls as frequently (N=73, P>0.05; n.s., not significant).

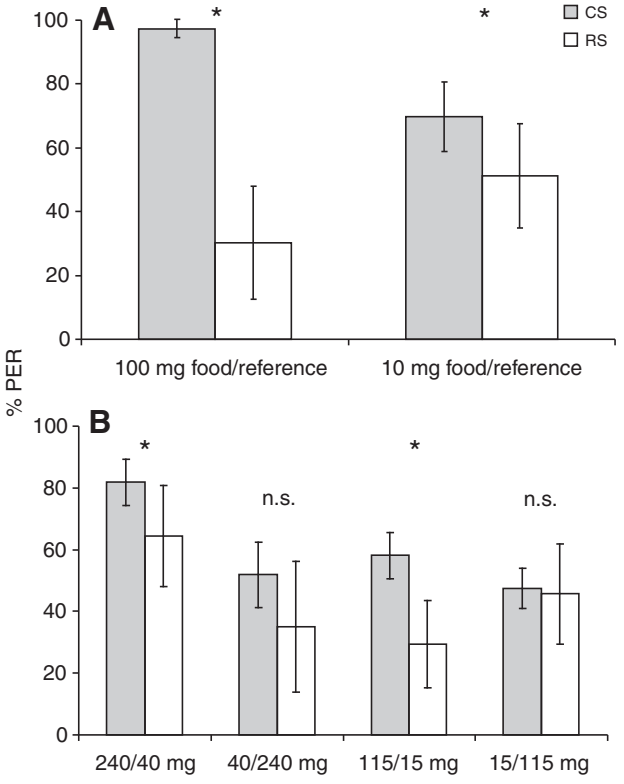

Fig. 3. Bees can be trained to respond to extracts of larval food by exhibiting the PER. (A) Conditioned responses to the odours of pentane extracts of 100 and 10 mg larval food were significantly greater than those to the solvent control stimulus (N=19 and 40, respectively, *P<0.05 in both series; Wilcoxon test). (B) The bees significantly prefer the higher concentration of larval food odour when trained to respond to the higher of two concentrations (N=20 in each series, *P<0.05; Wilcoxon test), but do not significantly prefer the lower concentration when trained to respond to the lower one (N=20 in each series, P>0.05; n.s.).

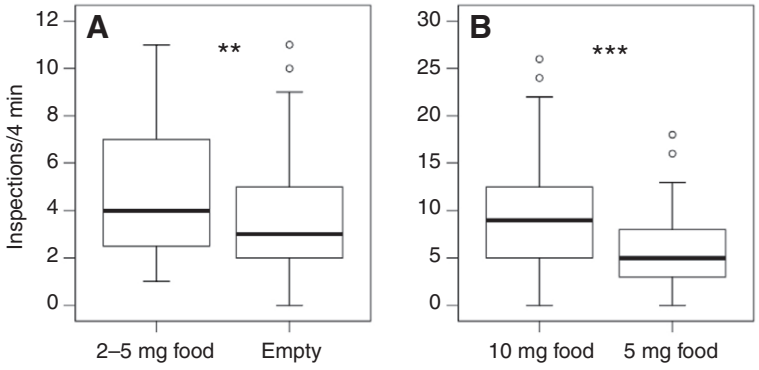

Fig. 4. Artificial 'cells' containing (A) a small amount (2–5 mg) of larval food are more attractive than empty cells (N=60 trials, $^{**}P$<0.01; Wilcoxon test) and cells containing (B) 10 mg larval food are more attractive than cells containing 5 mg food (N=87 trials, $^{***}P$<0.001; Wilcoxon test). Outliers indicated by open circles.

observed in a small test chamber containing cell-like structures containing larval food. 'Cells' with small amounts of food were clearly more attractive than empty cells (Fig. 4A), and cells containing a larger amount of larval food were significantly more attractive than those containing a smaller amount (Fig. 4B).

The principal components analysis of the gas chromatograms of pentane extracts of food-deprived and well-fed control larvae (Fig. 5) indicates that there are components available for olfactory discrimination between the signals of hungry and well-fed larvae.

In addition to chemical signals and cues, we analysed larval movements which might indicate the nutritional state of the larva by measuring the speed of movement of food-deprived larvae and a control group. Larval pace increased after 1–2 h of food deprivation (Fig. 6). After 3–4 h pace again complied with the control, and after 6 h the larvae moved slower than untreated ones. Immediately after feeding visits by worker bees, larval pace increased for about 3 min (P<0.05; Mann–Whitney U-test). Larvae also reacted on manual feedings with increased activity (P<0.01). Larval speed was positively correlated with the number of feedings (R=0.49/P<0.001; Spearman's rank-order correlation) and with the number of inspections by worker bees (R=0.45/P<0.001).

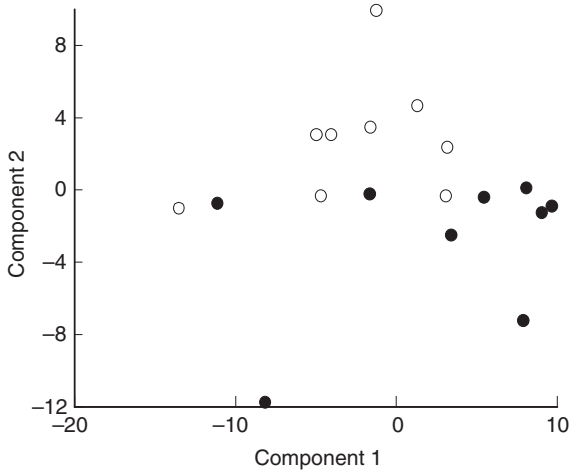

Fig. 5. Principal components analysis of the gas chromatograms of pentane extracts of food-deprived (filled circles) and well-fed control larvae (open circles). The analysis is based on 161 components of the extracts. Component 1 explains 29.5% of the variability, component 2 another 13.1%. Although there is a slight overlap between the samples taken from hungry and control larvae there seem to be components available for olfactory discrimination between the signals of hungry and well-fed larvae.

DISCUSSION

The data show that honeybee workers respond to the (experimentally induced) state of food deprivation of larvae. Huang and Otis (Huang and Otis, 1991a) have previously reasoned that food-deprived larvae were fed more often. We confirmed these results on the basis of a more precise definition of feeding visits.

This result indicates that there must be a mechanism of recognition of the food supplies or the nutritional state of the larvae. Such recognition has previously been demonstrated in ants (Cassill and Tschinkel, 1995; Kaptein et al., 2005) and bumble bees (Pereboom et al., 2003).

According to Huang und Otis (Huang and Otis, 1991a), experimental supply of food to starved larvae reduced their chance of receiving inspections longer than 10 s compared with unfed larvae. This is a further hint of food possibly being involved in the regulation of feeding behaviour.

However, we could not rule out the possibility that the behavioural changes of nurse bees observed by Huang and Otis (Huang and Otis, 1991a) and ourselves were caused not by food deprivation but by an artefact, i.e. the fact that the pieces of mesh material hindering the nurses from feeding the larvae additionally prevented bees in general from moving directly across these combs, which might have an effect on the smell of the combs afterwards. It is well known that tarsal pheromones are used to mark food sources in meliponines (Hrncir et al., 2004; Jarau et al., 2004), the nest entrance in *Vespula vulgaris* and the honeybee (Butler et al., 1969), and flowers in bumble bees (Goulson et al., 2000; Eltz, 2006) and honeybees (Stout and Goulson, 2001). We therefore improved the technique by

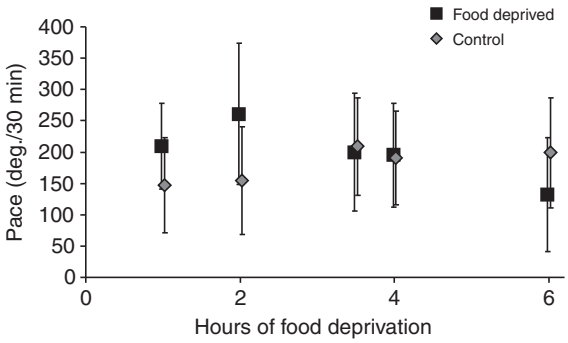

Fig. 6. Changes of larval pace following supply of food. After food deprivation, larvae show an initial increase in speed of rotation (N=5 trials; number of larvae per trial, 67±15 food deprived, 56±15 control; P<0.05, U-test). After 3–4 h pace again complies with the control (N=5 trials), and after 6 h (N=2 trials) the larvae move slower than untreated ones (P<0.05 in one of the two trials).

preventing the nurses from feeding the larvae without preventing them from walking on the cell rims. The results were indistinguishable from the effect of screening, indicating that indeed the changes within the cells of food-deprived larvae cause the intensified feeding behaviour.

As the gas chromatograph analysis revealed that the odour signals of food-deprived and well-fed larvae seem to be distinguishable, the question arose whether bees can indeed discriminate olfactorily between hungry and well-fed larvae. The results of the PER training clearly show that honeybees can at least learn to discriminate, and support similar results reported for bumble bees by den Boer and Duchateau (den Boer and Duchateau, 2006). However, the standard method of extraction of larval odours used in our study as well as in all comparable studies in the literature (Kubišova et al., 1982; Free and Winder, 1983; Le Conte et al., 1990; Garrido and Rosenkranz, 2004) does not exclude the possibility that the larval food rather than the larva itself is the carrier of the signal, for traces of larval food might 'contaminate' the extracts.

The results of the training experiments exhibit a clear asymmetry, an effect which has been described in previous PER studies (Laloi et al., 2000; Smith and Cobey, 1994; Laloi and Pham-Delègue, 2004; Châline et al., 2005). Pelz and colleagues (Pelz et al., 1997) reported that bees trained to discriminate between low and high concentrations of the same odour exhibit a similar asymmetry, responding at a much higher rate to a high concentration presented as the CS than to a low concentration used as the CS. The asymmetry in the results of our training experiments might thus reflect that it is a quantitative rather than a qualitative difference that nurse bees discriminate between hungry and well-fed larvae, and this might well be the amount of larval food present in the cell. In a more natural setting, an assay similar to the one used in experiments on the chemical orientation of *Varroa* (Rosenkranz, 1993), the bees clearly showed that they can spontaneously discriminate between amounts of larval food as found in the cells of well-fed fifth instar larvae and an amount that is 50% lower. Thus bees can monitor the amount of food available for the larva olfactorily.

Although it is entirely unclear whether and how bees can perceive the rotational movements of larvae we cannot rule out the possibility that in addition to the chemical cues identified here mechanical cues are used to monitor the nutritional state of the brood. There is information in the speed of rotation, but so far there is no direct evidence for the ability of worker bees to somehow measure the speed of movement of larvae.

We conclude that honeybees can indeed monitor the nutritional state of their brood and that the amount of available food is directly perceived and used for the purpose of an optimal just-in-time provisioning of the brood.

We thank Wolf Engels, who encouraged us to have a closer look at food provisioning in honeybees, Peter Rosenkranz, who kindly allowed us to use his gas chromatograph for the analyses, and Katrin Korczyk, Ina Lahnstein and Mareike Mucha, who helped with collecting part of the data.

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