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Research article

A larval hunger signal in the bumblebee *Bombus terrestris*S.P.A. den Boer^{1,2} and M.J.H.M. Duchateau^{2,*}¹ Copenhagen University, Institute of Biology, Department of Population Biology, Universitetsparken 15, 2100 Copenhagen, Denmark,
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Abstract. Larvae of *Bombus terrestris*, a pollen-storing bumblebee, are dependent on progressive provisioning by workers. We test the hypothesis that larval cuticular chemicals can act as a hunger signal. We first show with a new classical conditioning experiment, using a Y-shaped tube, that workers can be trained to prefer the extracts of normally fed larvae over those of starved larvae. This proves the ability of workers to discriminate between larval extracts. Second, we show in a bioassay that workers also use these perceived differences to feed larvae according to their nutritional status. Larval broods sprayed with the extracts of the starved larvae were fed significantly more than larval broods sprayed with the extracts of normally fed larvae or with the solvent (n-pentane) only. We therefore conclude that *B. terrestris* larvae signal their need for food via their cuticular chemicals, and discuss the extent to which this form of communication could give larvae some control over their development.

Keywords: *Bombus*, larval solicitation, hunger signal, cuticular chemicals, feeding.

Introduction

Social insect societies are characterised by obligate group living with overlapping generations and reproductive asymmetry between females of two different, phenotypically determined, castes (Michener, 1974). The queen caste lays the eggs and has direct reproduction. The worker caste performs colony tasks and increases its fitness through the relatedness with the brood they care for. Larvae of social Hymenoptera do not provide their own food but depend on worker nurturing. In stingless bees and 'primitive' ant subfamilies the lar-

vae can partially control their own food intake because food is provided by mass provisioning, meaning that food for the entire larval development is provided at once. In species like the honey bee, social wasps and ants the larvae are fed by progressive provisioning: the workers must regularly provide the larvae with food. This seems to give workers control over larval development by imposing the feeding regime upon these larvae (Bourke and Ratnieks, 1999).

In bumblebees both types of food provision are present. 'Pocket-makers' (e.g. *B. pascuorum*, *B. hortorum*, *B. diversus*) store pollen in separate pockets underneath brood clumps, creating a continuous food supply on which the larvae can feed directly. 'Pollen-storers' (e.g. *B. terrestris*, *B. hypnorum*, *B. terricola*) accumulate nectar and pollen in empty cocoons and specialised storage cells. These larvae depend on the workers to feed them progressively by regurgitating a mixture of nectar, pollen and glandular secretions (Michener, 1974; Pereboom, 2000). Each larva is thus fed individually (Van den Toorn and Pereboom, 1996), and the frequency of feedings is related to the progress in larval development (Ribeiro et al., 1999).

Several recent studies have challenged the prevalent view that workers of pollen storer species impose a feeding regime upon their larvae without any feedback (Plowright and Jay, 1977). In an experimental laboratory study, Pereboom et al. (2003) showed that starved *B. terrestris* larvae are fed significantly sooner and more often than well fed larvae. Smeets and Duchateau (2001) simulated larval provisioning by manually feeding larvae in a laboratory colony with a micropipette, and showed that these manually fed larvae subsequently received fewer feedings from workers than control larvae. In addition, hand-rearing experiments showed that larvae sometimes refuse food and thus cannot be forced to eat (Pereboom et al., 2003). These results indicate that larvae have a more active role in the feeding process and that the feeding behaviour of workers can be affected by the nutritional status of the larvae.

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To explain the results of these experiments Smeets and Duchateau (2001) and Pereboom et al. (2003) hypothesized that bumblebee larvae actively emit a hunger signal to which workers react. They showed that workers are able to perceive the presence of hungry larvae from a distance of up to 2 cm, which is normally sufficient when they work on the wax layers surrounding the larvae (Smeets and Duchateau, 2001). Such an active role of larvae in the feeding process has also been reported for other social insects. In the wasp *Vespa orientalis*, larvae actively beg for food by rhythmically rubbing their mandibles against the soil rings of the cell wall, thereby producing a scraping sound to which workers react by offering food (Ishay and Landau, 1972). Fire ant (*Solenopsis invicta*) larvae are fed at rates proportional to their body size and nutritional status and presumably solicit food with a non-volatile chemical (Cassill and Tschinkel, 1995).

Preliminary research on the nature and potential significance of hunger signals in *Bombus terrestris* has shown considerable differences in the concentrations of cuticular chemicals of starved and well-fed larvae (M.J. Duchateau, unpubl.). The objective of the present study was to investigate whether these larval cuticular chemicals could function as a hunger signal. We addressed this question using two different methods. First, a classical conditioning experiment was conducted, to determine whether worker bumblebees can discriminate between the cuticular extracts of starved and normally fed larvae. In honeybees, testing the proboscis extension reflex (PER) is a sound method for testing workers' ability to discriminate between odours (Bitterman et al., 1983), but studies on the PER in bumblebees showed that only a rather low percentage of workers could be trained (and with only a low number of responses) compared to honeybees (Laloi et al., 1999; Laloi, 2004). From this and our own experience we concluded that it would be rather difficult to condition bumblebees using PER. We therefore developed another method of classical conditioning using a Y-shaped tube. With this new method we can test if workers can discriminate between the larval extracts of normally fed and starved larvae after having been conditioned to one of them. Second, a bioassay was performed to test whether these larval extracts can act as a hunger signal and elicit differential feeding behaviour by workers, when sprayed on groups of larvae.

Material and methods

Extraction of larval cuticular chemicals

For the extraction of larval cuticular chemicals similar sized normally fed male larvae of about 5 days old were collected at random from a single colony reared in confinement (climate-controlled room, 28 °C and 60 % RH). Male larvae were used because of their abundance in older colonies that were present at that time. Individual larvae were immediately washed in 1 ml n-pentane (a solvent for lipid mixtures rich in hydrocarbons) for 15 min, and the same 1 ml n-pentane was reused for the successive extraction of cuticular chemicals of in total 10 male larvae. A second group of male larvae was removed from the same colony and returned to the colony in a glass tube covered with netting, making phys-

ical contact with and feeding by workers impossible, but maintaining exposure to colony odour and temperature. After a starvation period of 24 h cuticular chemicals of these larvae were extracted in the same way as for the normally fed larvae. In this manner, two different n-pentane solutions were obtained: one containing the cuticular chemicals of ten normally fed larvae and the other containing the chemicals of ten starved larvae.

Classical conditioning

All experiments were conducted under standard laboratory conditions in a climate-controlled room (28 °C, 60 % RH), illuminated with red light invisible to bees. Adult workers of unknown age were sampled from random colonies while they were nursing the brood. They were stored overnight in groups of 6–8 workers in small boxes with a limited supply of sugar solution to ensure survival, but also hunger so that the workers would be motivated to react to the conditioning rewards in the experiment. Some pupae, enclosed in cocoons, were added to the boxes to reduce stress (Smeets and Duchateau, 2001). In the subsequent experiments, workers were placed one by one in our standard experimental device consisting of a small circular chamber (Ø7.5 cm) connected to the long base arm of a 12.5 cm long Y-shaped plexiglas tube (Ø1.3 cm). Walking into this tube, workers had the choice between two short arms of 6 cm length (Ø1.3 cm) each of them terminating in a standard observation box containing some pupae enclosed in cocoons. In the short arms (at a distance of ±0.5 cm from the split) different larval cuticular extracts were presented, sprayed on small wax balls that were fixed inside the tubes (40 µl solution = 0.04 larval equivalent/ball). The wax was collected from the involucrum (isolating layer surrounding the brood nest) of a random colony and stored at –20 °C for a couple of days before use. In both arms the same wax was used, to prevent odour differences. Wax was chosen because in a natural colony larvae are surrounded by a wax envelope, which presumably is the carrier for the chemicals that they emit.

In classical (Pavlovian) conditioning a subject is trained to associate a novel stimulus (conditioned stimulus or CS) with a response (conditioned response or CR) by coupling this novel stimulus to a familiar stimulus (unconditioned stimulus or US) that is presented at the same time and to which the unconditioned response (UR) would normally be shown. The association between CS and CR is established by presenting a reward after each successful CR. After training, the subject should then be able to express the CR when only the CS is presented. In the Y-shaped tube in our experiment worker bumblebees are confronted with the cuticular extracts of starved larvae in one arm and the cuticular extracts of normally fed larvae in the other. The workers are rewarded after choosing the mixture of cuticular extracts of normally fed larvae (CS) and thus trained to choose the corresponding arm in the Y-shaped tube (CR) by coupling this CS to a familiar stimulus, the scent of honey (US) that is presented in the same arm. Preliminary research showed that bumblebee workers are highly attracted to the scent of honey. When a worker shows the correct response by walking into the arm in which the extracts of normally fed larvae are presented, she is rewarded with a drop of honey solution.

To condition the workers to recognize the extracts of normally fed larvae (CS) and enter the corresponding arm (CR), first a series of conditioning runs was performed. The conditioned stimulus was presented at random in the left and right arm of the Y-shaped tube to prevent location preference to become established. A drop of diluted honey solution was placed ca. 40 mm behind the wax ball containing the extracts of the normally fed larvae (CS). A worker was regarded to be trained to the conditioned stimulus if she had chosen the corresponding arm 5 times out of a maximum of 8 runs, allowing workers to make mistakes in the first few runs. After the conditioning runs, a series of test runs was performed to test the ability of workers to distinguish between the two larval extracts, without the presence of the US (scent of honey). In the test runs, each conditioned worker had to choose between the conditioned stimulus (extracts of normally fed larvae) and the stimulus they were not

trained on (extracts of starved larvae) in 8 separate runs, but this time without any reward present. After test-runs 3 and 7 two runs with a reward were executed to prevent extinction of the CR. The conditioned stimulus was again supplied randomly in either the left or the right arm, with clean tubes being provided after the reward runs.

One to four workers could be conditioned and tested on a single day. The time interval for individual workers between each single run was 15–90 min and we used series of eight runs to calculate the proportion of correct and incorrect choices. These proportions were paired for each of the tested workers and statistically analysed using a Wilcoxon signed rank test. Wilcoxon signed rank test is suitable, since it also takes into account the proportion of correct choices of each worker. To test the Y-shaped experimental setup itself, a control experiment was performed, applying the same cuticular extract in both arms. In these runs, workers were expected to choose the conditioned and unconditioned stimulus with the same frequency, unless unintended systematic differences would induce any bias. The results of these trials were also analysed with Wilcoxon signed rank test. A Mann-Whitney U test was used to compare the number of correct choices between the control experiment and the experiment using the larval extracts.

Bioassay

Small larval broods were created by placing 8 male larvae, all of similar size and age (about 5 days), and from the same colony, in small wax cups, which were then covered with a wax layer collected from the colony, where it was used as an isolation layer (= involucre) (Smeets and Duchateau, 2001). The larval broods were placed in a random colony overnight to allow workers to remodel the involucre, resulting in individual larval chambers around each larva. Three of these broods were used on each of the test days, and each brood sprayed with one of three different solutions: 100 µl of n-pentane (control), 100 µl of cuticular extract of starved larvae in n-pentane and 100 µl of cuticular extract of normally-fed larvae in n-pentane. The spraying was done in such a way that each larva within a brood was covered with fluid. The three broods were subsequently placed together in a small, young colony (containing 30–40 workers) and the number of feedings by workers to each larva in each brood was recorded during one-hour sessions. One to three one-hour sessions using the same broods with the same type of extracts could be performed each day. Before the start of each new session on the same day, the larval broods were taken out of the colony, sprayed again with the test and control solutions and returned to the colony. New larval broods were made on every new day of testing, and in total 18 one-hour sessions were conducted, in which the number of feeding was recorded. Typical feeding behaviour consists of the following characteristics: a worker bumblebee manipulates the wax envelope surrounding the larvae and makes a small hole in it with her mandibles (in last-instar larvae these openings are permanently open). The mandibles, antennae, and part of the head are inserted and after a short period of immobility the abdomen is lifted and a droplet of food is regurgitated from the crop onto the larva's ventral side.

For each brood, the number of feedings was averaged and expressed per larva per hour, and these averages were analysed using ANOVA with Tukey HSD as Post-Hoc test.

Results

Classical conditioning

Workers chose the conditioned stimulus (extracts of normally fed larvae) significantly more often than the unrewarding stimulus (extracts of starved larvae) (Fig. 1, right column; mean $\pm$ SEM: $77.3\% \pm 3.04\%$, Wilcoxon Signed Rank Test: $Z = -3.971$, $p < 0.001$, $n = 22$). However, when testing the

Y-maze set-up itself using the same cuticular extract in both arms instead of two different ones, workers showed a slight preference for the conditioned stimulus (Fig. 1, left column; $55.9\% \pm 2.65\%$, Wilcoxon Signed Rank Test: $Z = -1.999$, $p = 0.046$, $n = 17$). This indicates that the Y-maze system itself might have contained a bias that affected the choice of the workers. This bias was presumably caused by evaporated honey molecules that were retained on the wax balls. Nevertheless, the workers in the experiment using two different cuticular abstracts scored significantly better compared to those in the control experiment (Fig. 1; Mann-Whitney U test, $Z = -4.066$, $p < 0.001$, $n = 22$, 17). This implies that in the first experiment, besides the bias that the system has itself, there was an extra cue that enabled workers to choose correctly between arms: workers were able to distinguish between extracts of normally fed and starved larvae.

Bioassay

The larvae sprayed with the extracts of starved larvae received significantly more feedings per hour in comparison to the larvae that were sprayed with the extracts of normally fed larvae and to the control larvae. The latter two do not differ (Fig. 2; mean $\pm$ SEM: n-Pentane: 0.71 ± 0.09 , Normally-fed: 1.11 ± 0.14 , Starved: 1.77 ± 0.14 ; ANOVA, $F = 17.857$, $df = 2$, $p < 0.001$; Post Hoc Tukey HSD, n-Pentane control versus normally fed: $p = 0.113$, $n = 18, 18$; n-Pentane versus starved: $p < 0.001$, $n = 18, 18$; normally fed versus starved: $p = 0.001$, $n = 18, 18$).

Discussion

In social Hymenoptera larvae are dependent on workers for food and it would be advantageous for the larvae if they could give feedback on their need for food. The objective of

Figure 1. Mean percentage ($\pm$ SEM) of workers choosing the rewarded, conditioned stimulus (grey) and the unrewarded stimulus (white) during eight subsequent trials. The left bar represents the control experiment with similar larval extracts in both arms of the Y-shaped tube ($n = 17$; * = $p < 0.05$, see text). The right bar represents the experiment with the extracts of the normally fed larvae (conditioned stimulus) in one arm and the extracts of the starved larvae in the other ($n = 22$; ** = $p < 0.001$, see text). The horizontal bracket refers to the statistical comparison between both experiments (** = $p < 0.001$, see text).

Figure 2. Mean number of feedings per larva per hour ($\pm 95\%$ confidence interval) for the three different treatments. The brood sprayed with the extracts of starved larvae was fed significantly more (see text).

this study was to investigate whether larval cuticular washes can act as a hunger signal in the progressive feeding behaviour of workers of the bumblebee *Bombus terrestris*. The results of the classical conditioning experiment using the Y-maze set-up showed that most workers chose the extracts of normally fed larvae on which they were previously trained. From this we conclude that bumblebee workers are able to discriminate between the scents of normally fed and starved larvae. No direct contact between antennae and the wax balls sprayed with extracts was observed before the worker bees chose a direction in the Y-shaped tubes, which indicates that the larval cuticular extracts are at least low volatile and can be perceived from a distance of a few mm., which was also observed by Smeets and Duchateau (2001). These results also indicate that the Y-maze set-up is a good alternative to experiments using the proboscis extension reflex (PER), when testing odour discrimination in bumble bees.

A bioassay was conducted to test if worker bumblebees use these cuticular chemicals as an indication of the nutritional status of larvae and feed them accordingly. The number of feedings to larval broods sprayed with extracts of starved larvae (for 24 h), normally fed larvae and n-pentane (control) was recorded. Clear differences in feeding frequencies, were observed, suggesting that worker bumblebees can recognise hungry larvae by their cuticular chemicals and that these chemicals can act as a hunger signal. These cuticular chemicals are probably mainly hydrocarbons, because a) n-pentane is a solvent for lipid mixtures rich in hydrocarbons (Howard, 1993); and b) hydrocarbons are known to be widely involved in insect communication, for example as cues for nest mate recognition, sex discrimination, and caste recognition (Howard, 1993; Winston, 1992) but also in host-parasite interactions (Lenoir et al., 2001; Dronnet et al., 2005). These are all long-term recognition functions and the synthesis and spreading of these hydrocarbons probably takes as long as

the duration of a larval instar. The hydrocarbons that we hypothesize as hunger signal, must be expressed much more quickly. They would thus require a fast pathway from the oenocytes (cells belonging to the fat body, and in many insects an important site for hydrocarbon synthesis) to the cuticle, either by epidermal cells and pore canals, or via hemolymph transport (Young et al., 1999). Further studies on the short-term synthesis of volatile hydrocarbons of this kind would be needed to elucidate these questions.

An indication that larvae signal to be fed also comes from the work of Bortolotti et al. (2001), who induced the development of larvae into queens by topical application of JH early in colony development when normally only workers are produced. These queen-destined larvae were fed by the workers and actually developed as queens. There were no indications that these larvae could be forced to become workers by malnutrition, which would have been in the interest of the workers in that early stage of the colony development. All together, this indicates that larvae are more involved in the feeding process than was previously assumed by Plowright and Jay (1977), and that they do not just passively undergo their rearing. Whether the larval cuticular hydrocarbons act as a deliberate signal or a cue remains unclear from this study. The 'signal' interpretation would imply a significant cost and therefore honest signalling (Maynard Smith and Harper, 2003). The 'cue' interpretation would mean that the key hydrocarbons are accidentally released low-cost metabolites (Hauser, 1996), which trigger a response above a certain threshold (Hasson, 1994). The scraping behaviour of the hungry larvae of the wasp *Vespa orientalis* (Ishay and Landau, 1972) and the swaying behaviour of the larvae of the ant *Gnamptogenys striatula* (Kaptein et al., 2005), both resulting in feeding behaviour by workers, suggest at least some costs to the begging larvae. The enhanced feeding of starved larvae (Pereboom et al., 2003) and fewer feedings of manually fed larvae (Smeets and Duchateau, 2001) provide further evidence that honest signalling (Kilner and Johnstone, 1997; Rauter and Moore, 1999; Royle et al., 2002) exists in social insects. This would imply that feeding processes in insect societies can be seen as an action response game that results in a signalling equilibrium in which larvae and workers optimise their respective fitnesses (Maynard Smith and Harper, 2003).

If these signals are actively emitted and costly to the larvae, a potential conflict concerning caste determination could arise because female larvae might have the power to determine their own fate. Bourke and Ratnieks (1999) argued that queens, workers and developing female larvae are involved in the potential conflict about caste determination, each having different interests according to the kin selection theory. In this conflict the female larvae might derive selfish benefits from direct reproduction if they would develop as queens rather than workers, while the queen and workers would opt, in context of colony development, for the production of a bigger worker force. Bourke and Ratnieks (1999) hypothesised that the party that controls the nutrition of the larvae is likely to be the one determining female caste fate. Our present results now show that there is a direct feedback be-

tween larval hunger and feeding rate. When this feedback would consist of active signals, it might give the larvae some manipulative power and thereby some control over their development.

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