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ORIGINAL PAPER

Behavioral studies on tarsal gustation in honeybees: sucrose responsiveness and sucrose-mediated olfactory conditioning

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Abstract Although the forelegs of honeybees are one of their main gustatory appendages, tarsal gustation in bees has never been systematically studied. To provide a more extensive account on honeybee tarsal gustation, we performed a series of behavioral experiments aimed at characterizing (1) tarsal sucrose sensitivity under different experimental conditions and (2) the capacity of tarsal sucrose stimulation to support olfactory conditioning. We quantified the proboscis extension reflex to tarsal sucrose stimulation and to odors paired with tarsal sucrose stimulation, respectively. Our experiments show that tarsal sucrose sensitivity is lower than antennal sucrose sensitivity and can be increased by starvation time. In contrast, antennae amputation decreases tarsal sucrose sensitivity. Furthermore, we show that tarsal sucrose stimulation can support olfactory learning and memory even if the acquisition level reached is relatively low (40%).

Keywords Gustation · Honeybee · Tarsi · Sucrose · Olfactory learning

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Abbreviations

CS	Conditioned stimulus
US	Unconditioned stimulus
Gr	Molecular gustatory receptor
GRN	Gustatory receptor neuron
PER	Proboscis extension reflex
O	Group experiencing olfactory stimulation
T	Group experiencing tarsal sucrose stimulation

Introduction

Insects have largely contributed to our understanding of general principles of taste perception in animals, both at the periphery and at the central level (Dahanukar et al. 2005; Scott 2005; Hallem et al. 2006; Vosshall and Stocker 2007). In particular, the fruit fly *Drosophila melanogaster* has paved the way in research on insect gustation (Amrein and Thorne 2005; Scott 2005; Vosshall and Stocker 2007; Ebbs and Amrein 2007), especially since the decoding and publication of its genome (Adams et al. 2000). Indeed, the first taste receptor genes identified were those of the fruit fly (Clyne et al. 2000) and since then, 68 gustatory receptors (**Grs**) encoded by 60 genes through alternative splicing have been described (Dunipace et al. 2001; Scott et al. 2001; Robertson et al. 2003; Scott 2005), some of which have been linked to specific gustatory stimuli (Ueno et al. 2001; Dahanukar et al. 2001; Chyb et al. 2003; Marella et al. 2006; Moon et al. 2006).

Less is known, however, about taste in insects other than *Drosophila*, probably because complete genomic characterizations and the neurogenetic tools available for the fruit fly, which played a pivotal role in the characterization of taste perception, are so far inaccessible. It is therefore crucial to determine whether taste, as described for the fruit

fly, follows the same principles in other insects. The recent decoding and publication of the honeybee genome (The Honeybee Genome Sequencing Consortium 2006) raises doubts about the generality of the picture emerging from *Drosophila* studies. Instead of the 68 and 76 Grs found in the fruit fly and the related mosquito *Anopheles gambiae*, respectively, only ten gustatory receptors have been identified in the honeybee *Apis mellifera* by means of bioinformatics methods (Robertson and Wanner 2006). The gustatory world of bees was therefore considered to be limited when compared to that of flies and mosquitoes (Robertson and Wanner 2006).

This idea can be debated because even with ten gustatory receptors the dimensionality of taste perception can be highly complex if based on combinations of receptor inputs. Moreover, the complexity of the social life of bees, in which taste may play an important role for inter individual recognition, and the variety of natural gustatory compounds to which bees are exposed in different activities performed during their life raise some doubts about an eventual poorness of the bees' gustatory world (de Brito Sanchez et al. 2007). So far, few studies have studied gustatory perception in honeybees when compared to studies addressing the visual or the olfactory modality (Chittka and Brockmann 2005; Chittka and Raine 2006; Giurfa 2007). The antennae, mouth parts and tarsi of the forelegs constitute the main chemosensory appendages of bees (Goodman 2003). They include gustatory but also hygro-, thermo- and mechanosensory as well as olfactory sensillae. Taste sensillae have been characterized electrophysiologically at the level of the antennae (Haupt 2004; de Brito Sanchez et al. 2005) and mouthparts (Whitehead and Larsen 1976a, b; Whitehead 1978). A considerable number of behavioral studies have also concentrated on sucrose responsiveness when the antennae and mouthparts are stimulated (see Page et al. 2006 for review). More recently bitter taste has been also investigated at the antennal level (de Brito Sanchez et al. 2005). However, to our knowledge, only one brief study performed 70 years ago (Marshall 1935) has focused on tarsal gustation in honeybees. Marshall (1935) determined the concentration threshold of sucrose solution necessary to elicit the appetitive response of proboscis extension reflex (PER). He found that most of the bees exhibited PER at a concentration of 2.85% when stimulated at the antennae but that a concentration of 34% was required to elicit PER when the tarsi were stimulated.

Apart from studies on gustatory perception, PER has been extremely influential for the study of olfactory perception, learning and memory in honeybees (Guerrieri et al. 2005; Giurfa 2007). In these studies, the presentation of a neutral odorant that does not release PER in naïve animals is followed by sucrose stimulation, usually delivered to the antennae to elicit PER, and then to the proboscis. In this way, an association is formed which enables the odorant to release the PER in a following test. This effect is clearly associative and constitutes a case of classical conditioning (Bitterman et al. 1983), i.e., the odorant can be viewed as the conditioned stimulus (CS) and the sucrose solution as the rewarding, unconditioned stimulus (US). Within this framework, bees learn to associate the odorant with the sucrose reward. Several studies have dissected the differential contributions of antennal and proboscis US to olfactory and tactile learning in bees (Bitterman et al. 1983; Sandoz et al. 2002; Scheiner et al. 2005). These studies showed that sucrose solution applied to the proboscis determines the level of acquisition, whereas antennal input is generally of minor importance. Given that tarsal sucrose stimulation is able to elicit PER, it is important to determine the contribution of tarsal US input to olfactory learning in bees.

To provide a more extensive account on honeybee tarsal gustation, we performed a series of experiments aimed at characterizing (1) tarsal sucrose sensitivity under different experimental conditions (e.g., three levels of starvation, presence vs. absence of antennae), and (2) the capacity of tarsal sucrose stimulation to support olfactory conditioning. Our experiments confirm that tarsal sucrose sensitivity is reduced when compared to antennal sensitivity and show that it can change depending on factors such as starvation time and antennae amputation. We found low but consistent acquisition rates in olfactory conditioning based on tarsal sucrose stimulation alone, thus showing that tarsal reward can support associative learning in bees.

Materials and methods

Bees from a hive distant 50 m from the laboratory were captured and brought to the laboratory where they were cooled in ice for 3–5 min in order to facilitate their manipulation. Bees were captured when returning to the hive or when arriving at an artificial feeder in which sucrose solution was provided. In the former case, care was taken to avoid pollen foragers (bees with pollen on their back legs) as they possess different genetic background and highly variable sucrose sensitivity responses when compared to nectar foragers (Page et al. 2006).

Each bee was mounted into a small metal tube allowing only protrusion of the bee's head and fore tarsi. Thus, a harnessed bee could only move its antennae and mouthparts, including the proboscis. The forelegs were fixed wide open in order to facilitate their stimulation (Fig. 1). Unless specifically mentioned (e.g., experiments on the effect starvation on tarsal sucrose responsiveness), bees fixed in this way were fed with a drop of sucrose solution and kept for 2 h in a dark and humid container before the experiments. Stimulation was performed by means of a toothpick soaked

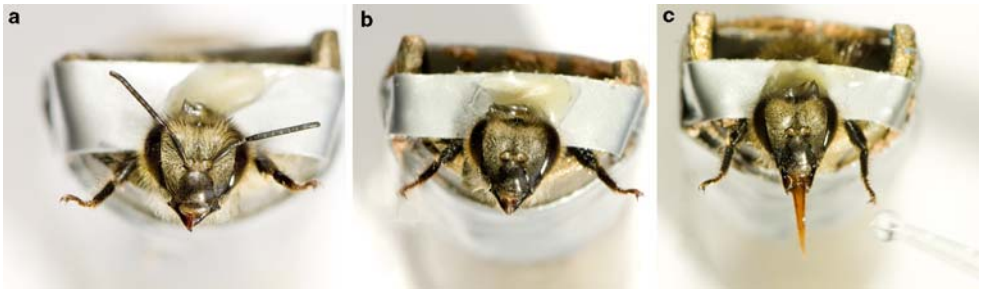

Fig. 1 **a** Intact honeybee harnessed in a metal tube with its forelegs protruding in order to allow tarsal gustatory stimulation. **b** Honeybee with amputated antennae in the same preparation. **c** Proboscis extension reflex upon tarsal stimulation in a bee with amputated antennae (photos by Cyril Frésillon @CNRS)

in the solution assayed. A different toothpick was used for each solution tested.

Experiment 1: Tarsal sucrose responsiveness

Individual responsiveness to sucrose was measured following Page et al. (1998) by touching the tarsi of the bees with the following six sucrose concentrations: 0.1, 0.3, 1, 3, 10 and 30%, which correspond to a logarithmic series of sucrose concentration of −1, −0.5, 0, 0.5, 1 and 1.5, respectively. Bees were always tested with an ascending order of sucrose concentrations to reduce potential sensitization that can occur with higher concentrations of sucrose. Stimulations with plain water were interspersed between sucrose stimulations in order to control for non-specific increases in responsiveness. The intertrial interval between two consecutive tests was 10 min. Stimulation of tarsi lasted 6 s and was performed on both tarsi consecutively. We recorded at which concentration a bee showed proboscis extension and counted for each individual the total number of proboscis responses to the six sucrose stimulations. This number is the '*gustatory response score*' of a bee. A bee responding to all six sucrose concentrations therefore has a gustatory response score of 6. A response was counted when a bee was observed to fully extend its proboscis beyond the virtual line between the mandibles (proboscis extension reflex or PER). Small movements of the proboscis that did not result in full extension were not considered to be responses.

In a *first series* we compared antennal versus tarsal stimulation in two groups of bees, one stimulated on the tarsi and the other stimulated on the antennae. This is similar to Marshall's (1935; see above) experiment, but in his study bees were not fully immobilized (they had just two slotted pieces of cardboard placed around the neck for separating head from tarsi) so that the use of a metallic holder in our case may change the responsiveness of the bees.

In a *second series* we compared three groups of bees for their tarsal sucrose responsiveness after different starvation times following fixation in individual harnesses. Bees were tested after starvation times of 30 min, 1 and 2 h. An independent group of bees was used in each case.

In a *third series* we compared tarsal sucrose responsiveness in two groups of bees, one in which the antennae were amputated and the other with intact antennae. Antennal amputation was performed to determine how two major chemosensory appendages interact and because Marshall (1935) argued that "*amputation of the antenna does not impair the normality of the bee in respect of its gustatory reactions*" but provided no data to support this statement. Amputation occurred at least 2–3 h before experiments. Both antennae were cut with fine scissors at the base of the scapus, taking care not to pull them. Bees with leaking hemolymph were eliminated from the analysis.

Experiment 2: Olfactory conditioning and tarsal sucrose stimulation

We investigated the capacity of tarsal sucrose reward to support olfactory conditioning in honeybees. We trained two groups of bees differing in their pairing of CS and US stimulations. In *Group OT* there were five trials in which odor (O) and tarsal US stimulations (T) were paired, and five interpolated blank trials; in *Group O/T* there were five trials with odor alone and five trials with tarsal US stimulation alone. Note that the blank trials in the OT group were used to equate the number of stimulations (5 O and 5 T) and trials (10) in these two groups. Thus, only the pairing between odor and US stimulations was different between the OT and the O/T groups. A comparison of performance between these two groups allows determining whether pairing odorant and tarsal sucrose stimulation supports olfactory conditioning.

Two odorants, 1-Nonanol and 2-Hexanol, were used throughout as conditioned stimuli (CS) in a balanced manner, i.e. for half of the bees in each group, 1-Nonanol was the CS, while for the other half, 2-Hexanol was the CS. These two odors were selected because they elicit low cross-generalization responses in appetitive olfactory conditioning in honeybees (Guerrieri et al. 2005). Fifty percent

(weight/weight) sugar solution (1.46 M) was used throughout as US. Only bees responding with PER to this solution delivered to the tarsi before conditioning were used in this experiment.

Each conditioning trial lasted 1 min. The bee harnessed in its individual tube was placed in an experimental holder with an air extractor placed behind it for 25 s to allow familiarization with the training situation. The air extractor impeded the accumulation of residual odors. Thereafter, the conditioning odorant (CS) was released for 6 s. Three seconds after CS onset, the tarsi were stimulated with sucrose solution (US), leading to a PER. Both stimulus overlap and interstimulus interval were therefore 3 s. The bee was left in the conditioning place during 29 s and then removed. In Group O/T, odor-alone trials followed the same sequence but no US was delivered. In the same group, tarsi-alone trials followed also the same sequence but this time no odorant was delivered. In the blank trials of Group OT, each bee was placed in the holder for 1 min without receiving any stimulation. The intertrial interval was always 10 min considering blank trials.

Bees trained with either CS (1-Nonanol or 2-Hexanol) were tested with both odorants 10 min after the last conditioning trial to assess the specificity of the olfactory memories evoked in retention tests. Retention tests were separated by 10 min. Half of the bees received the CS first and then the novel odorant while the other half experienced the reversed sequence. Odorant stimulation was identical to that of conditioning trials but no US was given after odorant delivery. In all cases, we recorded whether the bee extended its proboscis after onset of the odorant (CS) (conditioned responses). After retention tests, we checked for intact PER after tarsal US stimulation. Only bees exhibiting the reflex were kept for the analyses.

Statistics

We recorded PER to the sucrose stimulation (Experiment 1) or to the presented odorant (Experiment 2). Multiple responses during a single stimulation were counted as a single PER. The percentage of PER recorded was used to plot responsiveness (Experiment 1) and acquisition curves (Experiment 2). To analyze the variation of such a group performance during trials, we used analyses of variance (ANOVAs) for repeated measurements both for between-group and for within-group comparisons. Monte Carlo studies have shown that it is permissible to use ANOVA on dichotomous data under controlled conditions (Lunney 1970), which are met by our experiments (equal cell frequencies and at least 40 degrees of freedom of the error term). Gustatory response scores are individual scores and not a group performance. They were compared by means of a Mann–Whitney *U* test. Performances within a retention test were analyzed by means of a McNemar test.

Results

Experiment 1: Tarsal sucrose responsiveness

In a *first series* we compared two groups of intact bees ($n = 30$ each), one stimulated on the tarsi and the other stimulated on the antennae. Stimulation lasted 6 s and was performed on both tarsi consecutively or on both antennae simultaneously. This stimulation time ensured that sucrose was presented long enough to elicit consistent PER when delivered at the tarsi. Longer stimulation times did not change tarsal responsiveness (not shown), thus showing that possible differences between antennal and tarsal responsiveness were not due to technical differences in stimulating tarsi and antennae.

Figure 2a shows that antennal stimulation yielded a higher responsiveness than tarsal stimulation (ANOVA for repeated measurements: $F_{1,58} = 5.89$, $P < 0.02$). Bees in both groups significantly increased PER with increasing sucrose concentration ($F_{5,290} = 46.42$, $P < 0.0001$) and the interaction between the factors 'concentration' and 'stimulation site' was not significant ($F_{5,290} = 0.33$, $P = 0.90$). Responses to water were negligible and not significantly different between both groups of bees (Fig. 2a; factor trials: $F_{4,232} = 0.38$, NS; factor stimulation site: $F_{1,58} = 0.25$, NS). The difference between both groups can also be visualized by considering their respective responsiveness score, which is an individual's score calculated as the sum of all PERs made by a given bee along the whole scale of sugar concentrations (Fig. 3a). At the individual level, bees stimulated on the antennae had a significantly higher scores than bees stimulated on the tarsi (Mann–Whitney *U* test: $Z_{adj} = 2.13$, $P < 0.04$).

In a *second series*, we compared tarsal sucrose responsiveness after different starvation times. Three independent groups of intact bees were tested at 30 min ($n = 27$), 1 h ($n = 25$) and 2 h ($n = 28$) after fixation in individual harnesses. The latter starvation time is the one used in the previous series of experiments. Shorter times (30 min, 1 h) were chosen because preliminary experiments (not shown) showed that bees were less responsive to sucrose stimulation with shorter starvation times.

Figure 2b shows that in all three groups responsiveness significantly increased with sucrose concentration ($F_{5,385} = 60.17$, $P < 0.0001$). Moreover, responsiveness differed between groups ($F_{2,385} = 5.11$, $P < 0.01$) as bees with longer starvation times had higher sucrose responsiveness scores (Fig. 3b; Kruskal–Wallis test: $H_{2,80} = 9.04$, $P < 0.02$). Multiple comparisons yielded no difference between 1 h versus 2 h starvation ($P = 0.88$) and 1 h versus 30 min ($P = 0.22$). However, responses at 30 min were significantly lower than those at 2 h ($P < 0.02$). Responses to water stimulation interspersed between sucrose stimula-

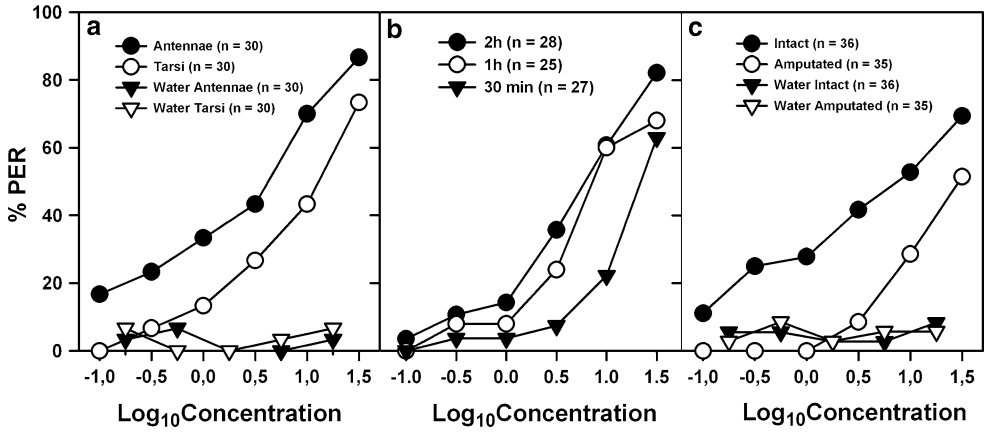

Fig. 2 The percentage (%) of honeybees that responded to increasing concentrations of sucrose solution with a proboscis extension reflex (PER). Sucrose concentrations have been transformed to $\log_{10}$ values. **a** Responsiveness to sucrose solution upon antennal (*black circles*) and tarsal stimulation (*white circles*). **b** Responsiveness to sucrose solution after different starvation times, 30 min (*inverted triangles*), 1 h (*white circles*) and 2 h (*black circles*). **c** The effect of antennal amputation on sucrose responsiveness (bees with amputated antennae: *white circles*; intact bees: *black circles*)

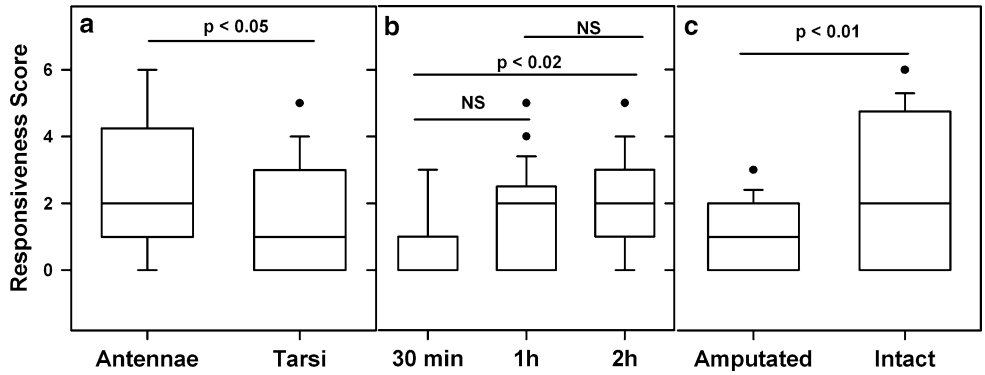

Fig. 3 Gustatory response scores (shown is the median and the 10th, 25th, 75th and 90th percentiles as *vertical boxes* with *error bars*). Black dots indicate outliers. For each bee, we counted the total number of proboscis responses to the first water and the six sucrose stimulations (see "Methods" for details). Bees responding to water and to all sucrose concentrations therefore have a gustatory response score of 7. **a** Scores of bees stimulated on the antennae and on the tarsi. **b** Scores of bees with starvation times of 30 min, 1 and 2 h. **c** Scores of intact bees and bees with amputated antennae

tions were negligible and not significantly different in all three groups (not shown; factor trials: $F_{4,304} = 0.32$, NS; factor starvation time: $F_{2,76} = 1.12$, NS). Sucrose was therefore the only stimulus causing higher responsiveness in bees that were starved longer.

In a *third series*, we compared tarsal sucrose responsiveness in two groups of bees, one in which the antennae were amputated ($n = 35$) and the other with intact antennae ($n = 36$). Figure 2c shows that although both groups increased their responses with sucrose concentration ($F_{5,345} = 36.21$, $P < 0.0001$), intact bees responded significantly more to sucrose than bees with amputated antennae ($F_{1,345} = 12.18$, $P < 0.001$). Therefore, amputated bees had significantly lower responsiveness scores than intact bees (Fig. 3c; Mann–Whitney U test: $Z_{adj} = 2.61$, $P < 0.01$). Neither amputated nor intact bees responded practically to water (Fig. 2c; factor trials: $F_{4,276} = 0.52$, NS; factor treatment: $F_{1,69} = 0.004$, NS).

In these three experimental series, three groups were in principle comparable because they were subjected to the same treatment: group 'Tarsi' in Fig. 2a, group '2 h' in Fig. 2b and group 'Intact' in Fig. 2c. They were all three constituted by intact bees that were stimulated with sucrose at the level of the tarsi after at least 2 h of starvation post fixation. It is thus interesting to compare their performances in order to detect the replication power of our procedure. There were neither significant differences between the responsiveness curves of these three groups n ($F_{2,364} = 1.79$, NS) nor a significant interaction ($F_{8,364} = 0.71$, NS), thus showing that despite the fact that our experimental series were fully independent, the performances of the groups subjected to identical treatments were the same.

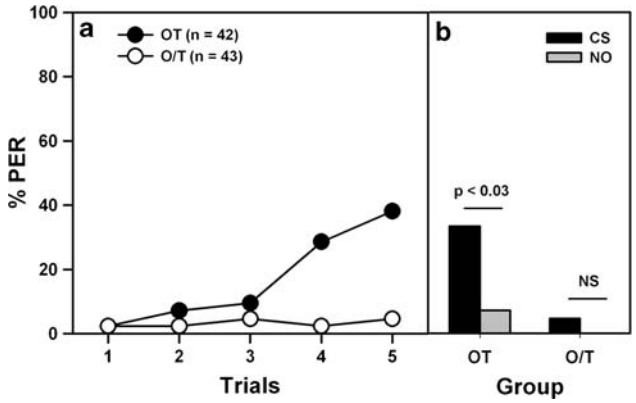

Fig. 4 The capacity of tarsal US to support olfactory learning and memory. In *Group OT* (*black circles*, *solid line*) there were five pairings of odor (O) and tarsal US stimulations (T), and five interpolated blank trials; in *Group O/T* (*white circles*, *solid line*) there were five trials with odor alone and five trials with tarsal US stimulation alone. Responses during blank trials were negligible and are not shown. **a** Acquisition curves. **b** Retention tests (no US provided) performed 10 min after the last acquisition trial. The response to the conditioned odorant (CS: *black bars*) and to a novel odorant (NO: *gray bars*) was recorded. In all cases the percentage (%) of PER is represented

Experiment 2: Olfactory conditioning and tarsal sucrose stimulation

Bees of the OT group were conditioned either with 2-Hexanol ($n = 20$) or with 1-Nonanol ($n = 22$). Sucrose stimulation was limited to the tarsi. There were no differences between these subgroups (2-factor ANOVA for repeated measurements: $F_{1,160} = 0.002$, NS) so that results were pooled. The performance of the OT group showed a significant increase of conditioned responses along trials (Fig. 4a: $F_{4,164} = 10.16$, $P < 0.001$) and reached a level of 38% correct choices in the last conditioning trial. Retention tests yielded similar results for bees trained with 2-Hexanol and 1-Nonanol (Fisher Exact Test: NS) and were therefore pooled. In such tests (Fig. 4b), bees specifically responded to the conditioned odorant and not to the novel odorant (McNemar Test: $\chi^2 = 9.09$, df 1, $P < 0.03$) but the levels of response to the trained odorant remained low (33%).

Bees of the O/T group, presented either with 2-Hexanol ($n = 22$) or with 1-Nonanol ($n = 21$), experienced five trials with odor alone and five trials with tarsal US stimulation alone. There were no differences between subgroups ($F_{1,164} = 0.11$, NS) so that results were pooled. Bees of the O/T group did not show any increase in their responses to the odorant along trials (Fig. 4a: $F_{4,168} = 0.21$, NS). In the tests they did not respond to either odorant (Fig. 4b), thus showing that no learning occurred under these experimental conditions.

Comparison between groups OT and O/T was highly significant both for acquisition ($F_{1,332} = 15.46$, $P < 0.0002$) and retention ($F_{1,83} = 12.56$, $P < 0.001$), thus showing that contingency between an olfactory stimulus and tarsal US is able to support olfactory learning and retention even if the level of correct responses reached is relatively low.

Discussion

Our study provides a behavioral account of honeybee tarsal gustation, an area of study that has been neglected for over 70 years in this insect. Our experiments show that, in the experimental conditions of the present work, tarsal sucrose sensitivity is lower than antennal sensitivity and can be increased by starvation. In contrast, antennae amputation decreases tarsal sucrose sensitivity. Finally, we show that tarsal sucrose stimulation can support olfactory conditioning per se.

Tarsal and antennal sucrose responsiveness

Marshall (1935) found that bees exhibited PER at a concentration of 2.85% when stimulated at the antennae but that a concentration of 34% was required to elicit PER when the tarsi were stimulated. Our results are consistent with this estimation as they show that over a wide range of sucrose concentrations sucrose responsiveness is always significantly higher for antennal than for tarsal stimulation (Fig. 2a). A mechanistic basis for this difference could be found at the level of the peripheral organs for taste detection, the taste sensilla, existing on the antennae and the tarsi. Based on ultrastructural descriptions of these sensilla (sensilla chaetica and basiconica), Whitehead and Larsen (1976a) reported 318 sensilla chaetica but no sensilla basiconica on the antennae and 10–20 sensilla chaetica and 0–6 sensilla basiconica per tarsomere of the forelegs. Thus, a simple numeric comparison shows that, at least for sensilla chaetica, the antennae are equipped with 15–30 times more receptors than the tarsi, a fact that could be related to the higher responsiveness for sucrose evinced upon antennal sucrose stimulation. Such a comparison is, however, senseless without an accurate functional characterization of the specificity and sensitivity of tarsal taste receptor cells by means of electrophysiological recordings. Such data are so far not available for the honeybee tarsi.

The effect of starvation on tarsal sucrose responsiveness

Sucrose responsiveness is not static as it can be modified by environmental and internal factors affecting appetitive motivation. Our results show that 1 h starvation determines an increase in tarsal sucrose responsiveness especially for sucrose concentrations higher than 3% (see Fig. 2b). This result underlines the importance of controlling starvation

times when measuring appetitive responses. Page et al. (1998) had shown that sucrose responsiveness of bees stimulated at the level of the antennae changes depending on feeding experiences. Responsiveness is logically higher before feeding and considerably lower after feeding (Page et al. 1998; see their Fig. 1). In the fruit fly *Drosophila melanogaster*, sucrose responsiveness was assessed using tarsal stimulation as in our work (Scheiner et al. 2008). In this case, food deprivation strongly modulated responsiveness to different sucrose concentrations. After 2 h of food deprivation, sucrose responsiveness was lower than after 24 h of food deprivation. Here we show that significant variations can occur within shorter starvation periods, a fact that can be related to the cellular processes controlling sucrose responsiveness. Indeed, the biogenic amines octopamine and tyramine can enhance sucrose responsiveness in the range of minutes (Scheiner et al. 2002). Octopamine is a key substance for motivational control as it modifies neural function at multiple levels, leading to a general sensitization of multiple behaviors (Libersat and Pflüger 2004; Huber 2005). One could therefore imagine that starvation increases octopaminergic activity, thus leading to higher appetitive responsiveness.

The effect of antennal amputation on tarsal sucrose responsiveness

The fact that amputation of the antennae reduces sucrose responsiveness seems to be logical given that the bees have been subjected to a harmful procedure that may interfere with the production of an appetitive response. Marshall (1935) argued that "amputation of the antenna does not impair the normality of the bee in respect of its gustatory reactions" but he provided no data to support this statement, which was unfounded in the light of our results. Two possibilities, which in fact are not mutually exclusive, can be invoked to account for these results. On one hand, the drop in response could be mediated by "discomfort" resulting from injury and by costs in wound repairing processes, or, on the other hand, it could it be that removal of a major chemosensory organ has more specific effects (e.g., removing any baseline neuronal input to higher centers of gustation, either inhibitory or excitatory) on tarsal gustation. So far, it is not possible to decide between these options. Nevertheless, our finding has a critical implication in the field of honeybee learning and memory. Recently, a new protocol for conditioning harnessed bees with visual stimuli paired with sucrose solution has been made available (Hori et al. 2006, 2007) based on work by Kuwabara (1957). The critical feature for such a visual conditioning to occur is amputation of the antennae but the mechanistic reasons for this remain unknown. Although in this conditioning protocol bees are directly stimulated with sucrose at the level of the proboscis, it is to be expected that antennal amputation reduced their general responsiveness to sucrose stimulation even after prolonged recovery times. This would explain why acquisition levels are always low in visual conditioning of harnessed bees (plateau around 40%). In this case, since acquisition is estimated using a group measure (% proboscis extensions), such a percentage reflects the fact that acquisition is good in few individuals but poor in the rest (after amputation), thus creating an overall (group level) impression that learning is poor. We argue that avoiding amputation in individuals with reduced sucrose responsiveness should improve acquisition levels in such protocol.

Olfactory conditioning based on tarsal sucrose stimulation

Despite inducing a lower responsiveness, tarsal sucrose stimulation was able to support olfactory conditioning per se, without delivery of sucrose to the proboscis. Tarsal sucrose stimulation was used in the first protocols of visual and olfactory conditioning in honeybees (Kuwabara 1957; Takeda 1961). In the first case, bees were trained to associate a chromatic stimulus (the conditioned stimulus or CS) with sucrose reward (the unconditioned stimulus or US), and in the second case they learned to associate an olfactory stimulus (CS) with sucrose reward (US). In the original protocols, sucrose was delivered to the tarsi, in order to elicit PER, and then to the proboscis (Kuwabara 1957; Takeda 1961). The US was therefore a compound signal (tarsi + proboscis). Later, the tarsal stimulation was replaced by antennal stimulation followed by sucrose to the proboscis (Bitterman et al. 1983). A fundamental question in conditioning is how much excitatory strength is supported by each US component. In olfactory conditioning, Bitterman et al. (1983) showed that the feeding component of the US plays a major role while the antennal US component has a minor role in conditioning despite the fact that it is very effective in eliciting PER. Indeed, Bitterman et al. (1983) showed that after four trials of pairing between odor and antennal US only, a level approximately 30% correct choices was attained while adding the US to the proboscis (the feeding component) enhances conditioned responses to 80–90% correct choices (see Fig. 4 in Bitterman et al. 1983). Here we showed that the levels of olfactory acquisition and retention based on tarsal US stimulation alone are also low but nevertheless significant. Comparison between groups OT and O/T showed that only the group OT in which the olfactory stimulus was contingent with the tarsal US exhibited some acquisition and retention. These two groups had exactly the same sensory experiences and differed only in the contingency between olfactory stimulation and tarsal US. Taken together our results show that tarsal US will support some conditioning but not a very high

level despite the fact that the sucrose concentration delivered at the tarsi is extremely effective in eliciting PER.

Perspectives

In this study we have focused on sucrose gustation at the tarsal level. Further studies are necessary to characterize tarsus-mediated responses in different behavioral contexts as one cannot exclude that the tarsal taste system is used in contexts in which feeding and PER responses are only marginal (e.g. resin collection, avoidance of bitter substances or pheromone detection via contact chemoreception). Research on tarsal taste in other insects such as the locust, the fruit fly and some butterflies has shown that tarsal taste receptors may be involved in contexts very different from feeding. In the locust, for instance, chemical stimulation of contact chemoreceptors located on the legs evokes withdrawal movements of the leg (Gaaboub et al. 2005). The likelihood of withdrawal depends on the leg considered (fore vs. hind leg), the site of stimulation (distal vs. proximal) and on the identity and concentration of the chemical stimulus used for stimulation (Rogers and Newland 2000). In the fruit fly, three types of tarsal sensilla have been found in females against only two types in males (Meunier et al. 2000). Such a sexual dimorphism is in part related to the perception of pheromonal compounds but also to the perception of sugar in the case of the female-specific sensilla type, which is absent in males (Meunier et al. 2000). In the buttefly *Papilio xuthus*, females are strongly deterred from laying eggs on the rutaceous plant *Orixa japonica* by two major compounds present in the leaves, characterized as 5-[[2-*O*-(beta-D-apiofuranosyl)-beta-D-glucopyranosyl]oxy]-2-hydroxybenzoic acid and adisyringoyl aldaric acid, and detected via the tarsi (Ono et al. 2004). These examples show the extent to which tarsal taste may be involved in contexts different from feeding but nevertheless essential for individual survival.

Related to this point, tarsal responses to other tastants such as salts, water and bitter substances need also to be characterized in the honeybee. The latter are particularly interesting since behavioral experiments have reported improvement in visual discrimination performances when one visual target is rewarded with sucrose and an alternative target is associated with quinine (Chittka et al. 2003; Dyer and Chittka 2004a, b). Rejection of bitter nectar by bees has also been reported in a more naturalistic setting (Johnson et al. 2006). As electrophysiological and behavioral experiments failed to detect a sense for bitter at the level of the antennae (de Brito Sanchez et al. 2005) and mouth parts (de Brito Sanchez et al., in preparation) the tarsi may be the structure on which sensilla containing bitter receptor neurons are located. Further electrophysiological experiments should study whether dedicated gustatory receptors neurons for bitter taste can be found on the honeybee tarsi.

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