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Responsiveness to sucrose and habituation of the proboscis extension response in honey bees

Received: 19 February 2004 / Revised: 6 May 2004 / Accepted: 6 May 2004 / Published online: 5 June 2004
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Abstract In honey bees, complex behaviours such as associative learning correlate with responsiveness to sucrose. In these behaviours, the subjective evaluation of a sucrose stimulus influences the behavioural performance. Habituation is a well-known form of non-associative learning. In bees, the proboscis extension response can be habituated by repeatedly stimulating the antennae with a low sucrose concentration. A high sucrose concentration can dishabituate the response. This study tests whether habituation correlates with responsiveness to sucrose in bees of different behavioural states and in bees which are habituated with different sucrose concentrations. Habituation and dishabituation in newly emerged bees, 5-day-old bees and foragers strongly correlated with responsiveness to sucrose. Bees with high responsiveness to sucrose displayed a lower degree of habituation and showed greater dishabituation than bees with low responsiveness. The degree of habituation and dishabituation also depended on the concentration of the habituation stimulus. These experiments demonstrate for the first time in a non-associative learning paradigm that the subjective strength of a sucrose stimulus determines the behavioural performance. Non-associative learning shares this property with associative learning, which suggests that the two processes might rely on similar neural mechanisms.

Keywords Behaviour · Habituation · Honey bee · Proboscis extension response · Responsiveness to sucrose

Abbreviations: GRS: Gustatory response score · PER: Proboscis extension response

Introduction

The sucrose concentration of a foraging site determines several aspects of foraging behaviour, such as the size of the crop load of foragers (von Frisch 1965; Pflumm 1969; Schmid-Hempel et al. 1985; Núñez and Giurfa 1996) and the probability and intensity of recruitment dances (von Frisch 1965; Raveret-Richter and Waddington 1993; Seeley 1995; Waddington 1997). The bees of a colony can vary greatly in their responsiveness to sucrose stimuli, independent of their nutritional state (for review see Scheiner et al. 2004). Individual responsiveness to sucrose can be measured using the proboscis extension response (PER). When the antennae of a bee are stimulated with a sucrose solution of sufficient concentration, the bee reflexively extends its proboscis in expectation of food. Individual responsiveness to sucrose is measured by applying a series of sucrose concentrations to the antennae of a bee. Highly responsive bees respond to sucrose concentrations as low as 0.1% or even to water, whereas unresponsive bees only respond to 30% sucrose or higher sucrose concentrations (for review see Scheiner et al. 2004).

Individual responsiveness to sucrose allows some predictions on the responsiveness to stimuli of other sensory modalities. Bees with high responsiveness to sucrose are more sensitive to pollen and have lower response thresholds for an appetitive odour, measured in an olfactometer, than bees with low responsiveness to sucrose (Scheiner et al. 2004).

Individual responsiveness to sucrose is an important determinant in the division of foraging labour. Water foragers are the most responsive bees. Pollen foragers are also very responsive. Bees that collect both nectar and pollen display an intermediate responsiveness to sucrose. Nectar foragers have a comparatively low responsiveness to sucrose (Pankiw and Page 1999, 2000). Even among the group of nectar foragers responsiveness to sucrose differs between individuals. Nectar foragers which are highly responsive to sucrose

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collect nectar with lower sugar concentrations than those that are less responsive (Pankiw and Page 2000; Pankiw 2003).

Another important correlate of responsiveness to sucrose is associative learning. The performance of a bee in associative tactile or olfactory learning strongly correlates with individual responsiveness to sucrose. Bees displaying high responsiveness to sucrose reach higher asymptotes of their acquisition function than bees with low responsiveness to sucrose (Scheiner et al. 1999, 2001a, b, c, 2003a). Memory retention measured 24 h after conditioning is stronger in bees with high responsiveness to sucrose (Scheiner et al. 2004). Differences in the associative tactile and olfactory learning performance of bees of different genetic strains can be explained by differences in their responsiveness to sucrose (Scheiner et al. 2001a, b). Pollen collecting foragers perform better in associative learning than nectar foragers, because they are more responsive to sucrose (Scheiner et al. 1999, 2003a).

Non-associative and associative learning are accomplished by similar neural mechanisms (Hawkins and Kandel 1984). These findings give rise to the question whether even non-associative learning, such as the inhibition of the PER, is dependent on responsiveness to sucrose, similar to associative learning. If this is true, individual differences in responsiveness to sucrose should result in different degrees of habituation, which can be tested in appropriate experiments.

So far, associative learning has not been demonstrated in newly emerged bees, and non-associative learning has never been tested in newly emerged bees. If these young bees show indeed habituation of the PER, non-associative and associative learning would develop in different time scales in honey bees.

Habituation has already been investigated in much detail (Thompson and Spencer 1966), which makes it a well-suited example of non-associative learning. In studies on habituation of the PER in honey bees, it has been assumed that a solution of a particular sucrose concentration would have the same effect on all bees (Braun and Bicker 1992; Bicker and Hähnlein 1994; Guez et al. 2001; Lambin et al. 2001). In associative learning, on the other hand, it has already been shown that the effect (i.e. the learning level) depends on the individual's responsiveness to sucrose. In the present study it is tested whether even non-associative learning, i.e. habituation, depends on individual responsiveness to sucrose. The question is examined in two different ways. Firstly, it is tested whether or not different bees are habituated as fast as others by repeated encounters with sucrose solution of a particular concentration. Secondly, it is analysed whether or not a higher sugar concentration is necessary in some bees than in others in order to achieve a particular degree of habituation. In the former case the sucrose concentration is constant. In the latter case it varies.

Another question which is addressed in this study is whether newly emerged bees can learn non-associatively.

This has never been tested. To my knowledge, all attempts to condition newly emerged bees associatively have failed. The general assumption is that brain structures which are important for learning are not fully developed in very young bees. It will be interesting to see whether non-associative learning performance changes from newly emerged bees over 5-day-old bees to foragers and whether possible differences in habituation are related to the well-known age-dependent changes in responsiveness to sucrose (Pankiw and Page 1999).

Materials and methods

Preparation of the bees

Honey bee brood combs were stored in an incubator until bees started emerging. Newly emerged honey bees were brushed off the combs every 3 h. Part of these bees were immediately mounted in small holding tubes as described in Erber et al. (1998). Strips of adhesive tape were attached between head and thorax. This group is later referred to as "newly emerged bees". Some of the bees were paint-marked after emergence and added to a small colony. These bees were retrieved from the hive after 5 days, immobilized in a refrigerator and mounted. Returning nectar foragers were caught at the entrance of the hive after blocking the entrance with a wire mesh. These bees represented a typical foraging cohort and were of mixed ages. After immobilization, the foragers were mounted in the holding tubes like the other bees.

One hour after mounting, responsiveness to sucrose was tested using the PER. Water and the following six sucrose concentrations were applied to the antennae of each bee: 0.1, 0.3, 1, 3, 10 and 30% (w/v). With each sucrose concentration it was recorded whether proboscis extension occurred. The inter-trial interval was 2 min. The sum of the responses to water and the six sucrose concentrations represents the gustatory response score (GRS) of an individual and is a measure of its responsiveness to sucrose (Scheiner et al. 2004). GRSs ranged between 0 (no response to water or any of the sucrose solutions) and 7 (PER to all solutions).

The role of responsiveness to sucrose and behavioural state in habituation

To analyse the effects of responsiveness to sucrose and behavioural state on habituation of the PER, individuals of three behavioural groups were tested for habituation: newly emerged bees, 5-day-old bees and returning nectar foragers. Ten seconds after measuring the concentration dependence of the PER, the first series of 30 antennal stimulations with 1% sucrose ("habituation" trials) started. Animals which did not show proboscis extension in the first stimulation were discarded. Thus, response levels at the beginning of the habituation experiment were equal in all groups. Normally, one

would not expect animals with very low responsiveness to sucrose to respond in the first trial, because the sucrose concentration of the habituation stimulus (1% sucrose) was below their sucrose response threshold. But the last sucrose stimulus applied during measuring the concentration dependence of PER was 30% sucrose and this stimulus was applied 10 s before the first series of 1% sucrose stimulations started. This high sucrose concentration was supposed to sensitise the bees. Therefore, a number of bees with very low GRSs could be tested for habituation. At each sucrose stimulation it was recorded whether a bee responded with proboscis extension. The sum of PERs in the 30 sucrose stimulations constitutes the “habituation score” of a bee for the first habituation phase. Because only animals which responded in the first trial were analysed, habituation scores ranged from 1 to 30.

Ten seconds after the 30 antennal stimulations with 1% sucrose, bees were stimulated 5 times with 30% sucrose to induce dishabituation. The sum of responses to these five sucrose stimulations represents the “dishabituation score” of an animal. Ten seconds later, a second series of 30 antennal stimulations with 1% sucrose followed. The sum of responses to the 30 sucrose stimulations constitutes the “habituation score of the second habituation phase”. The inter-stimulus interval was 10 s throughout the habituation experiment.

Habituation using different sucrose concentrations

In this experiment, foragers whose responsiveness to sucrose had been tested were repeatedly stimulated with one of the following sucrose concentrations: 0.1, 1% sucrose (this is the same group as was tested in the study involving different behavioural states) or 10% sucrose. Repeated antennal sucrose stimulation with one of the three sucrose concentrations (“first habituation phase”) started 10 s after testing the concentration dependence of PER. The protocol for habituation was the same as described above, apart from using different sucrose concentrations as habituation stimuli. The five dishabituation trials were conducted with 30% sucrose; the “second habituation phase” was performed in the same way as the “first habituation phase”, again involving different habituation stimuli for the different groups.

Behavioural measures and statistics

To analyse the relationships between responsiveness to sucrose, behavioural state, and the degree of habituation and dishabituation, three values were calculated from experimental results: (1) GRSs, (2) habituation scores and (3) dishabituation scores (see above). The GRSs have already been shown to constitute a reliable measure of responsiveness to sucrose (Scheiner et al. 2004). Habituation scores mirror the degree of habituation (Fig. 1); dishabituation scores display the degree of

dishabituation. A low habituation score implies a high degree of habituation and vice versa. A high dishabituation score shows a high degree of dishabituation and vice versa.

The effects of responsiveness to sucrose and behavioural state on habituation and dishabituation (first experiment) were analysed using analysis of variance (ANOVA). Habituation scores of the first and the second habituation phases and dishabituation scores were tested for correlation with GRSs and with behavioural state. Although some of the groups did not show normally distributed habituation and dishabituation scores, ANOVA was used, because it is very robust against departures from normality (Lunney 1970; Zar 1999; Bortz et al. 2000). The Scheffé test was used as post hoc test (Zar 1999). In the second experiment, the effects of responsiveness to sucrose and of the sucrose concentration used as habituation stimulus (0.1, 1 and 10% sucrose) on habituation scores and dishabituation scores were analysed using ANOVA. The Scheffé test was used as a post hoc test.

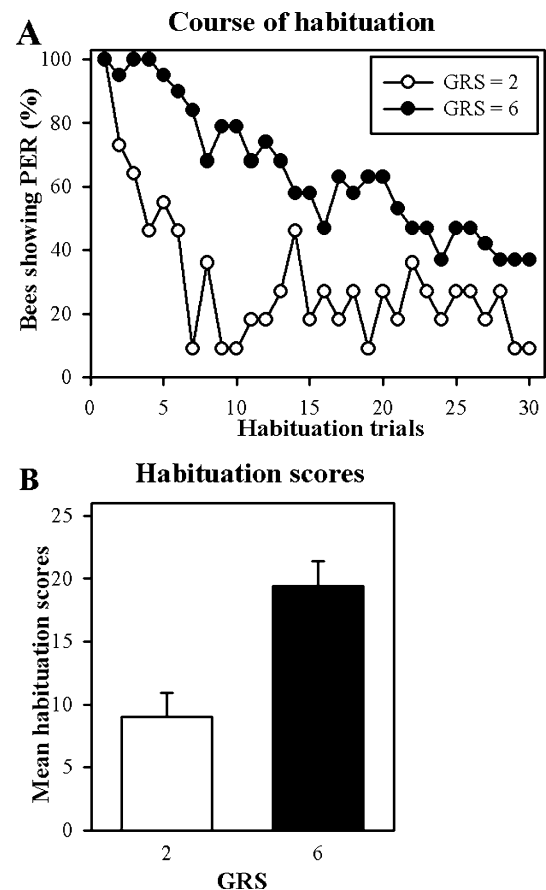


Fig. 1a, b Habituation scores represent the degree of habituation. The proboscis extension response (PER) of newly emerged honey bees was habituated using a 1% sucrose solution. **a** Course of habituation of two groups of bees differing in their responsiveness to sucrose, measured as gustatory response scores (GRSs). **b** Mean habituation scores of the same bees and standard errors of the mean. Number of animals tested: $n_{GRS=2} = 11$, $n_{GRS=6} = 19$

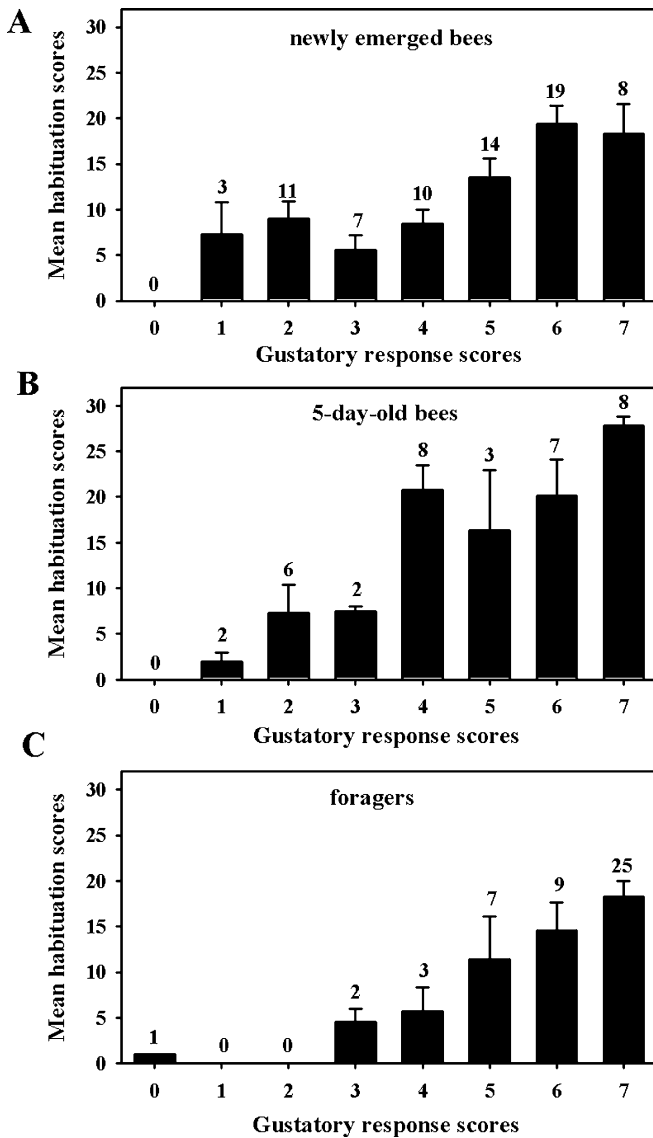


Fig. 2a–c Correlation between GRSs and habituation scores in honey bees of different behavioural states. **a** Newly emerged bees (1–4 h old), **b** Five-day-old bees taken out of a hive, **c** Returning nectar foragers. The number of bees tested is given for each GRS class. Data are mean ± standard error of the mean (SEM) of habituation scores for different GRS classes

Results

The degree of habituation correlates with responsiveness to sucrose

The typical course of habituation of the PER is shown in Fig. 1a. With increasing number of sucrose stimulations, the percentage of bees showing proboscis extension decreases. The degree of habituation can be expressed as habituation score, which comprises the total number of responses to the 30 sucrose stimulations. The habituation scores in Fig. 1b were calculated as mean values of the poorly responsive bees (GRS = 2) and of the highly

responsive bees (GRS = 6) of Fig. 1a, each separately. It is evident that the inhibitory effect of the habituation procedure is much more effective in the former group than in the latter.

In the three behavioural groups, the degree of habituation depended not only on responsiveness to sucrose, measured as GRSs but also on the behavioural state of the animals [GRS: $F_{(7,154)} = 8.72$, $P \leq 0.001$; behavioural state: $F_{(2,154)} = 3.55$, $P \leq 0.05$; ANOVA]. In all behavioural groups, bees with high GRSs displayed higher habituation scores than bees with low GRSs (Fig. 2). Behavioural state did not show a consistent effect on responsiveness to sucrose (Fig. 2). However, newly emerged bees had significantly lower habituation scores than 5-day-old bees ($P \leq 0.05$, Scheffé test). Foragers showed intermediate habituation.

After 30 stimulations with 1% sucrose all bees were stimulated with 30% sucrose for five times to induce dishabituation. In all behavioural groups, this sucrose concentration led to a high degree of dishabituation. Dishabituation scores (see Materials and methods) were affected by responsiveness to sucrose [$F_{(7,154)} = 3.64$, $P \leq 0.001$, ANOVA] but not by behavioural state (Fig. 4a). Bees with high responsiveness to sucrose responded more often in the dishabituation trials than bees with low GRSs. The differences in dishabituation scores were particularly great between the groups with very low GRSs (GRS classes 0 and 1) and those with higher GRSs (GRS > 1).

Response levels after the five dishabituation trials were approximately 80% in all three behavioural groups. This demonstrates that bees of all three behavioural states were dishabituated to a similar degree and that responsiveness at the beginning of the second habituation phase was overall approximately 20% lower than at the beginning of the first habituation phase.

The five stimulations with 30% sucrose were immediately followed by a second series of 30 stimulations with 1% sucrose. In this “second habituation phase”, habituation scores (not illustrated) were again affected by responsiveness to sucrose [$F_{(7,154)} = 3.75$, $P \leq 0.001$] and by behavioural state [$F_{(2,154)} = 3.50$, $P \leq 0.05$, ANOVA].

Habituation in bees with different GRSs using different sucrose concentrations

The effect of different sucrose concentrations used as habituation stimuli on the degree of habituation was tested in foragers. These bees were tested for their responsiveness to sucrose and subsequently stimulated 30 times with 0.1, 1 or 10% sucrose.

Habituation scores were affected by both responsiveness to sucrose [$F_{(7,147)} = 7.28$, $P \leq 0.001$] and the sucrose concentration of the habituation stimulus [$F_{(2,147)} = 3.67$, $P \leq 0.05$, ANOVA]. In all three groups, individuals with high GRSs displayed higher habitua-

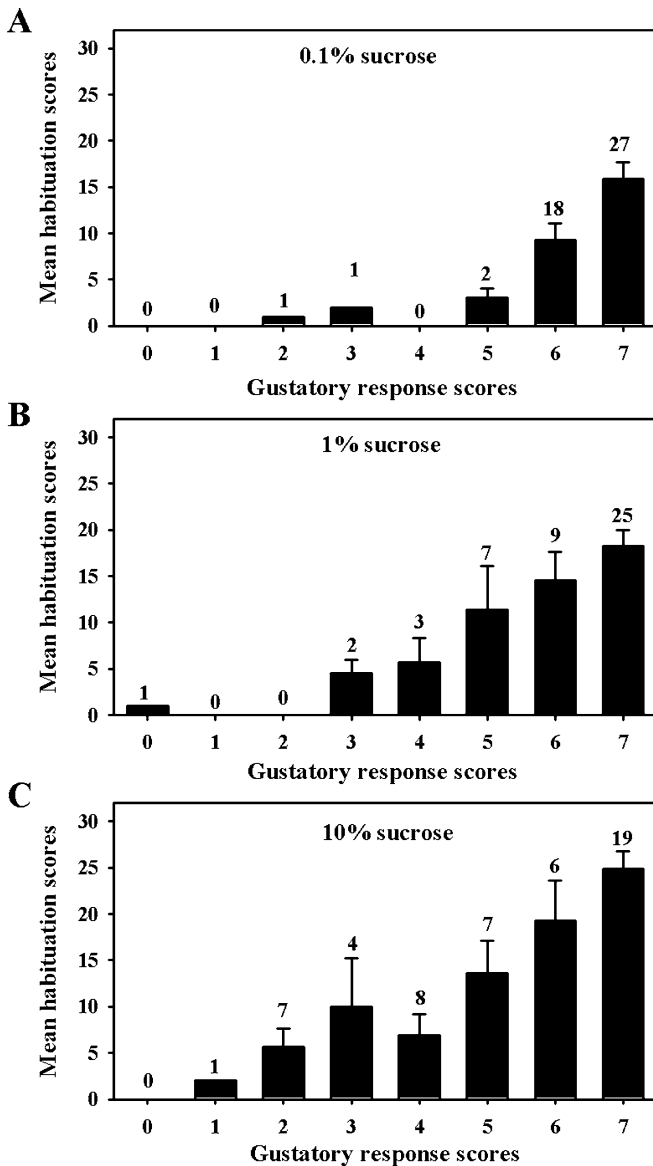


Fig. 3a–c Correlation between GRSs and habituation scores in honey bee foragers which were repeatedly stimulated with different sucrose concentrations. **a** Stimulation with 0.1% sucrose. **b** Stimulation with 1% sucrose. **c** Stimulation with 10% sucrose. The number of bees tested in each group is given. Data are mean + SEM of habituation scores for different GRS classes

tion scores than bees with low GRSs (Fig. 3). The higher the sucrose concentration of the habituation stimulus, the higher were the habituation scores in the different GRS classes (cf. Fig. 3a–c).

After 30 habituation trials, all bees were stimulated with 30% sucrose for five times to induce dishabituation. Dishabituation scores were affected both by responsiveness to sucrose [$F_{(7,147)}=3.35$, $P \leq 0.01$] and by the sucrose concentration of the habituation stimulus [$F_{(2,147)}=13.35$, $P \leq 0.001$, ANOVA]. The correlation between dishabituation scores and responsiveness to sucrose was particularly strong in the group which was habituated with 10% sucrose (Fig. 4b) and less pro-

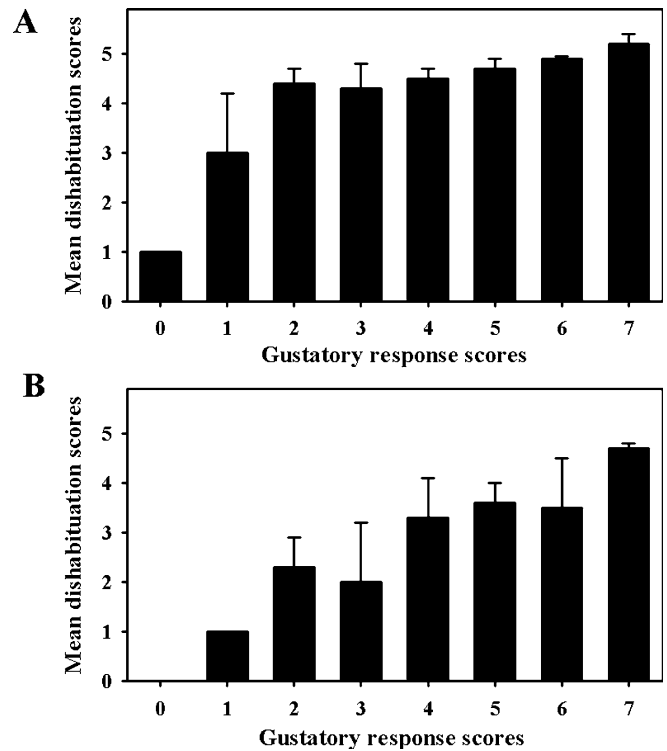


Fig. 4a, b Dishabituation scores of bees in different GRS classes. During the dishabituation phase, bees were five times stimulated with 30% sucrose. Data are mean + SEM. **a** For this figure the data of newly emerged bees, 5-day-old bees and foragers which had previously been stimulated with 1% sucrose in the habituation phase were pooled, because the behavioural state did not affect the correlation between responsiveness to sucrose and dishabituation. **b** Dishabituation scores of foragers which had been stimulated with 10% sucrose in the habituation phase

nounced in the other groups (not illustrated). Response levels after the five dishabituation trials were approximately 80% in the groups which had been habituated using 0.1 or 1% sucrose. Bees which had been habituated with 10% sucrose only responded to 67% after the five dishabituation trials.

After the five dishabituation trials, a second series of 30 habituation trials was conducted using the same three sucrose concentrations as before. As before, responsiveness to sucrose and the concentration of the habituation stimulus affected habituation scores [GRS: $F_{(7,147)}=3.90$, $P \leq 0.001$; concentration of habituation stimulus: $F_{(2,147)}=3.10$, $P \leq 0.05$, ANOVA].

Discussion

These experiments demonstrate a strong correlation between responsiveness to sucrose and habituation of the PER in honey bees. In newly emerged bees, in 5-day-old hive bees and in foragers, habituation correlated with individual responsiveness to sucrose. Bees with high responsiveness to sucrose showed less habituation than bees with low responsiveness to sucrose.

The second factor which determined the degree of habituation was the sucrose concentration used as habituation stimulus (compare, for example, the corresponding GRS classes between Fig. 3a and c). In bees of the same GRS classes repeated stimulation with a high sucrose concentration induced a lower degree of habituation than stimulations with a lower concentrated sucrose solution. This result fits well in the habituation theory of Thompson and Spencer (1966). The current results additionally demonstrate that a defined habituation stimulus can lead to very different degrees of habituation, depending on the subjective perception of this stimulus. A high sucrose concentration used as habituation stimulus did not have the same subjective value for each individual. Depending on individual responsiveness to sucrose, a 10% sucrose solution could result in stronger habituation than a 0.1% sucrose solution (compare, for example, GRS class 4 in Fig. 3c with GRS class 7 in Fig. 3a). This finding is very important, because it implies that the same sucrose concentration used as habituation stimulus can have very different meanings for different bees. In associative learning, it was shown that the difference between the sucrose concentration at the individual response threshold and the sucrose concentration of the reward determined individual learning performance. A similar rule is true for non-associative learning. The degree of habituation of the PER depends on the individual perception of the habituation stimulus. The subjective value of a habituation stimulus can be expressed as the difference between the individual perception threshold for sucrose and the sucrose concentration of the habituation stimulus. The importance of individual responsiveness to sucrose for both habituation and associative learning suggests similar mechanisms underlying both forms of learning. In order to equalize experimental conditions for bees, the sucrose concentration of a habituation stimulus should be chosen depending on the individual responsiveness to sucrose.

Habituation also correlated with the behavioural state of a bee. Five-day-old bees had significantly higher habituation scores than newly emerged bees. This is consistent with the finding that responsiveness to sucrose increases with age (Pankiw and Page 1999). Five-day-old bees, which usually have a higher responsiveness to sucrose than newly emerged bees, should also habituate more slowly, because habituation correlates with responsiveness to sucrose. Based on the study of Pankiw and Page (1999) one would expect that foragers should be even more responsive to sucrose and should therefore show a still lower degree of habituation. However, in my experiments I found no difference in the habituation of foragers and 5-day-old bees. This finding could be related to the fact that nectar foragers were tested after their return from a foraging trip. Most of these bees presumably had filled honey stomachs, which strongly reduces responsiveness to sucrose (Page et al. 1998). This could have led to lower habituation scores.

Age studies on the habituation of the PER have been conducted before. Guez et al. (2001) showed that young bees (4–7 days old) needed fewer trials for habituation than older bees (8–10 days old). The current findings can explain the results of Guez et al. (2001). Because responsiveness to sucrose generally increases with age (Pankiw and Page 1999), the faster habituation of the young bees was probably related to the lower responsiveness to sucrose of these bees. The current results can also explain the findings of Braun and Bicker (1992), who showed that hungry bees needed more trials for habituation than fed bees. Hungry bees usually have a higher responsiveness to sucrose than satiated bees (Page et al. 1998) and should therefore need more trials for habituation.

The present results demonstrate that responsiveness to sucrose is a good indicator for the sensory responsiveness of honey bees. Responsiveness to odours and to pollen, and foraging behaviour have been shown to correlate with responsiveness to sucrose (for review see Scheiner et al. 2004). Associative and non-associative learning correlate with responsiveness to sucrose, which shows in both cases that the individual evaluation of the reward or of the habituation stimulus determines to a large degree the behavioural performance. Once the GRS of an individual has been determined, reliable predictions on diverse and complex behaviours, such as learning and division of labour, of this animal can be made.

Taken together, the correlations between responsiveness to sucrose, responsiveness to other stimulus modalities and diverse behaviours suggest that many behaviours correlate with the general sensory responsiveness of an individual bee. Whether sensory responsiveness is causally linked to these behaviours is unclear, because so far, only correlations between responsiveness to sucrose and behavioural scores have been shown. It would be interesting to look at the behaviour of animals after manipulating their sensory responsiveness.

How sensory responsiveness is regulated is only partially known. An important part of individual responsiveness to sucrose is fixed genetically (Page et al. 2000; Page and Erber 2002). Bees selected over many generations for “high-pollen-hoarding” behaviour (Page and Fondrk 1995) are significantly more responsive to sucrose than bees selected for “low-pollen-hoarding” behaviour (Page et al. 1998; Pankiw and Page 1999; Scheiner et al. 2001b). In these strains and in wild type bees, pollen foragers are always more responsive to sucrose than nectar foragers (Page et al. 1998; Pankiw and Page 1999, 2000; Scheiner et al. 2001b, 2003a). Several quantitative trait loci have now been found which correlate with responsiveness to sucrose and with the decision to collect pollen or nectar (Page and Erber 2002). The differences between pollen and nectar foragers and between the two genetic strains are therefore not a result of their different nutritional states. Responsiveness to sucrose shifts from hungry to satiated individuals in a similar way in pollen and nectar foragers and in the two genetic strains (Page et al. 1998). For that reason,

satiated pollen foragers are still more responsive to sucrose than satiated nectar foragers and satiated bees of the “high-pollen-hoarding” strain are still more responsive to sucrose than satiated bees of the “low-pollen-hoarding” strain (Page et al. 1998). The changes in responsiveness to sucrose which occur throughout the year (Scheiner et al. 2003a), the changes related to different ages (Pankiw and Page 1999) and the changes related to nutrition and experience (Pankiw et al. 2001) must have a different source. The cellular substrate underlying responsiveness is unknown. First studies indicate a role of protein kinases (Scheiner et al. 2003b) and of biogenic amines (Scheiner et al. 2002). But this aspect still awaits further investigation.

One important aspect of responsiveness to sucrose is that foraging behaviour correlates with it. Pollen foragers are on average more responsive to sucrose than nectar foragers (Page et al. 1998; Pankiw and Page 2000; Scheiner et al. 2001b, 2003a). In how far these differences in sensory responsiveness are linked to the learning behaviour of these bees during their foraging trips is unclear. Based on the current results, nectar foragers should habituate faster than pollen foragers, because they are less responsive to sucrose. This hypothesis can be tested experimentally under laboratory conditions. In the future, new experiments have to be designed to also test sensory responsiveness to different stimulus modalities in relation to learning and other behaviours in free-flying pollen and nectar foragers.

Acknowledgements I would like to thank Siegrid Wiese for her help with the experiments and Joachim Erber for his helpful advice on this manuscript. This work was supported by a grant from the Deutsche Forschungsgemeinschaft (SFB 515). All experiments conducted comply with the current German guidelines for the proper treatment of animals.

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