The Journal of Experimental Biology 215, 1076-1083

doi:10.1242/jeb.063297

RESEARCH ARTICLE

Octopamine improves learning in newly emerged bees but not in old foragers

Andreas Behrends[1] and Ricarda Scheiner[1,2,*]

[1]Technische Universität Berlin, Institut für Ökologie, 10587 Berlin, Germany and [2]Universität Potsdam, Institut für Biochemie und Biologie, Zoophysiologie, 14476 Potsdam, Germany

*Author for correspondence (Ricarda.Scheiner-Pietsch@Uni-Potsdam.de)

Accepted 31 October 2011

SUMMARY

Honey bees (*Apis mellifera*) are well known for their excellent learning abilities. Although most age groups learn quickly to associate an odor with a sucrose reward, newly emerged bees and old foragers often perform poorly. For a long time, the reason for the poor learning performance of these age groups was unclear. We show that reduced sensitivity for sucrose is the cause for poor associative learning in newly emerged bees but not in old foragers. By increasing the sensitivity for sucrose through octopamine, we selectively improved the learning performance of insensitive newly emerged bees. Interestingly, the learning performance of foragers experiencing the same treatment remained low, despite the observed increase in sensitivity for the reward. We thus demonstrate that increasing sensitivity for the reward can improve the associative learning performance of bees when they are young but not when they had foraged for a long time. Importantly, octopamine can have very different effects on bees, depending on their initial sensory sensitivity. These differential effects of octopamine have important consequences for interpreting the action of biogenic amines on insect behavior.

Key words: honey bee, PER, olfactory conditioning, ageing, sucrose responsiveness.

INTRODUCTION

Biogenic amines are important modulators of behavior in vertebrates and invertebrates. Dopamine, serotonin and histamine are present in both vertebrates and invertebrates. The catecholamines norepinephrine and epinephrine have only been found in vertebrates, whereas the phenolamines octopamine and tyramine have similar physiological functions in invertebrates (for reviews, see Roeder, 2005; Scheiner et al., 2006).

Model invertebrate organisms such as honey bees or fruit flies obviously present great advantages for studying the function of biogenic amines because their brains are less complex than those of vertebrates and they are easily accessible for different techniques. Importantly, biogenic amines seem to have similar physiological properties in vertebrates and invertebrates. Honey bees are perfect organisms with which to study the functions of biogenic amines in behavior, because they have a huge behavioral repertoire even under controlled laboratory conditions.

Of the biogenic amines studied in this manuscript, dopamine plays a central role as a mediator of punishment in aversive learning, as has been shown in experiments with fruit fly mutants (Schwärzel et al., 2003; Schroll et al., 2006), crickets (Unoki et al., 2005) and honey bees (Vergoz et al., 2007). In appetitive learning, dopamine has rather inhibitory effects on acquisition or retrieval (Mercer and Menzel, 1982; MacMillan and Mercer, 1987; Menzel et al., 1990; Menzel et al., 1999). Besides, dopamine was shown to decrease sensitivity for sucrose in honey bee foragers (Scheiner et al., 2002), although its function for sensory sensitivity in other age groups has not been studied.

Among other effects, serotonin has been shown to reduce sensory sensitivity to odors, water vapor or high sucrose concentrations (for a review, see Erber et al., 1993), but the effects on sensitivity for lower sucrose concentrations have not been tested. In addition, serotonin reduces the number of bees showing conditioned responses after conditioning (Mercer and Menzel, 1982).

Behavioral data on the function of tyramine in honey bees and other insects are scarce. The few available studies suggest that tyramine is involved in honey bee motor behavior (Fussnecker et al., 2006). In addition, the tyramine receptor mutant *honoka* displayed reduced olfactory sensitivity (Kutsukake et al., 2000). In honey bee foragers, tyramine increases sensitivity for sucrose (Scheiner et al., 2002) and increases the rate of habituation (Braun and Bicker, 1992).

Of the biogenic amines studied in insects, octopamine has received the most attention. This amine was shown to increase peripheral olfactory sensitivity in moths (Pophof, 2002) and honey bees (Mercer and Menzel, 1982; Menzel et al., 1988; Spivak et al., 2003) (for reviews, see Erber et al., 1993; Scheiner et al., 2006). In honey bee foragers, octopamine increases sensitivity for sucrose (Scheiner et al., 2002). This amine is particularly important for associative appetitive learning in insects, because it mediates the reward signal (Schwärzel et al., 2003; Unoki et al., 2005; Schroll et al., 2006; Vergoz et al., 2007). Hammer (Hammer, 1993) showed at the cellular level that octopamine mediates the reward signal in olfactory learning in honey bees. A ventral unpaired median neuron (VUM_{mx1}) that belongs to a group of octopamine immunoreactive neurons and has its soma in the suboesophageal ganglion depolarizes in response to sucrose stimulation. Current injection into this neuron can substitute for the sucrose reward during olfactory conditioning. This suggests that during associative training, this neuron releases octopamine, which then mediates the reward signal (Hammer, 1993; Hammer, 1997). Further, injections of this amine rescue the proboscis extension response and appetitive conditioning in bees depleted of biogenic amines (Menzel et al., 1990; Menzel et al., 1999; Braun and Bicker, 1992). Recently, disruption of an

octopamine receptor by RNA interference was shown to reduce associative learning performance (Farooqui et al., 2003).

Honey bees have a long history as model systems for learning and memory because they learn fast and reliably even under controlled laboratory conditions and develop a stable memory over days (Kuwabara, 1957; Bitterman et al., 1983; Sandoz et al., 1995; Menzel and Müller, 1996; Scheiner et al., 1999; Scheiner et al., 2001a; Scheiner et al., 2001b; Scheiner et al., 2004; Scheiner et al., 2005; Giurfa, 2007; Wright et al., 2007). One of the classic learning experiments uses the proboscis extension response (PER). When the antennae of a bee are stimulated with a droplet of sucrose solution, the bee reflexively extends her proboscis in expectation of food. This behavior can be paired with an odor. If the bee experiences the odor shortly before her antennae are stimulated with sucrose solution, and if the bee is subsequently allowed to drink from the sucrose solution, she learns to extend her proboscis in response to the odor (Kuwabara, 1957; Bitterman et al., 1983; Sandoz et al., 1995; Wright et al., 2007). Honey bees even learn to associate an odor when the sucrose solution is only applied to their antennae (Sandoz et al., 2002). Similarly, bees can be trained to show proboscis extension in response to a tactile structure presented to their antennae (Scheiner et al., 1999; Scheiner et al., 2001a; Scheiner et al., 2005).

Individual sensitivity for sucrose, which can be measured using the PER, appears to be the major motivational factor in tactile and olfactory PER learning of honey bees. Pollen foragers usually learn an odor or a tactile stimulus faster than nectar foragers because they are more sensitive to the rewarding sucrose solution (Scheiner et al., 1999; Scheiner et al., 2001b; Scheiner et al., 2003). Even satiated pollen foragers are more sensitive to sucrose than respective nectar foragers (Page et al., 1998). Bees of two genetic strains that were selected for the amount of pollen stored in the colony (Page and Fondrk, 1995) have been shown to also differ in their olfactory and tactile learning performance, because they differ in their sensitivity to sucrose (Scheiner et al., 2001a; Scheiner et al., 2001b). Bees of the high-pollen-hoarding strain are usually more sensitive to low sucrose concentrations than bees of the low-pollen-hoarding strain (Pankiw and Page, 1999) and, accordingly, they learn better tactile and olfactory cues, independent of whether the bees had been foraging (Scheiner et al., 2001a; Scheiner et al., 2001b).

When wild-type nectar foragers that were very diverse in their sensitivity to sucrose were trained using equal subjective rewards (i.e. bees with low sensitivity received a highly concentrated sucrose solution and bees with high sensitivity were rewarded with a low sucrose concentration), they did not differ in their learning performance, which stresses the importance of individual sensitivity for sucrose for learning success (Scheiner et al., 2005).

We studied whether increasing sensitivity to sucrose by biogenic amines can improve associative learning performance in poor learners. There are different ways to address this question. One is to satiate bees. This decreases their sensitivity for sucrose (Page et al., 1998) and reduces their learning performance in appetitive learning (Ben-Shahar and Robinson, 2001; Friedrich et al., 2004). Another is to test age groups of bees with poor learning performance (Behrends et al., 2007; Scheiner and Amdam, 2009; Tolfsen et al., 2011). Obviously, the mechanisms underlying the poor learning performance of these groups must be different.

We focused on age-dependent poor learning performance and studied newly emerged bees and foragers with a long foraging duration. These groups are particularly interesting. They both perform poorly in associative olfactory learning. However, the mechanisms leading to low acquisition scores are different, because newly emerged bees are usually insensitive to low sucrose concentrations whereas old foragers are very sensitive to sucrose (Behrends et al., 2007; Scheiner and Amdam, 2009; Tolfsen et al., 2011). In addition, foragers with long foraging activity display higher levels of oxidative damage in the brain than younger bees (Seehuus et al., 2006). Our experiments test whether the same biogenic amine pathways are responsible for the poor learning performance in newly emerged bees and in foragers with long foraging experience. As we have previously shown that biogenic amines can have different effects on sensitivity for sucrose in foragers (Scheiner et al., 2002), we first tested which of the amines dopamine, serotonin, octopamine and tyramine would increase sensitivity for sucrose in newly emerged bees. Then we studied how an increased sensitivity would affect olfactory learning performance.

MATERIALS AND METHODS

Bees

To obtain newly emerged honey bees (*Apis mellifera* L.), frames with sealed brood shortly before eclosion were incubated at 34°C and 70% humidity. Emerging brood was brushed off the combs into a collection pan. The bees were given 30 min to accustom to light and the new environment. For collection of hive bees of different age groups, newly emerged bees were paint-marked at their thorax, restored to a colony with a naturally mated queen and collected off the frames 6 or 12 days after emergence. Each hive bee was collected individually and transported in a small glass vial.

Foragers with long foraging durations (‘old foragers’) were collected at the hive entrance when they returned from a foraging trip. Before, foraging activity of the entire colony had been observed for several weeks and foragers returning to the hive had received a paint mark on their thorax. We could therefore collect foragers with foraging durations >15 days, but we did not measure entire foraging duration or age as this has been shown to be unnecessary based on earlier studies on bees with long and short foraging durations (Behrends et al., 2007; Scheiner and Amdam, 2009).

At capture, foragers were individually placed in small glass vials. All bees apart from newly emerged bees were cooled in a refrigerator maintained at 4°C until they showed first signs of immobility. All bees were then mounted in small plastic holders as described previously (Bitterman et al., 1983; Scheiner et al., 1999; Scheiner et al., 2001a; Scheiner et al., 2001b; Scheiner et al., 2004; Scheiner et al., 2005). Briefly, a strip of adhesive tape between the head and the thorax fixed them in place while allowing them to move their antennae and mouthparts. A second strip of tape was fixed over the abdomen to prevent the bees from stinging. After mounting, all bees were left undisturbed for 1 h in a humidified chamber.

Measuring sensitivity for sucrose

To determine individual sensitivity for different sucrose concentrations, the antennae of each bee were touched with a droplet of water or sucrose solution of increasing concentrations as described previously (Scheiner et al., 2005; Behrends and Scheiner, 2009; Scheiner and Amdam, 2009). We recorded proboscis extension following stimulation of the antennae with the following solutions: water, 0.1% sucrose, 0.3% sucrose, 1% sucrose, 3% sucrose, 10% sucrose and 30% sucrose. The sum of proboscis extensions to stimulation with seven different solutions constitutes the gustatory response score (GRS) of a bee. These gustatory scores correlate with sucrose response thresholds of individuals and are a good estimate of individual sucrose responsiveness (Scheiner et al., 2004; Scheiner and Erber, 2009). Bees with a GRS of 2 or less were

Table 1. Treatment groups of newly emerged honey bees, concentrations of amines and number of bees tested

Treatment	Concentration (mol l^{-1})	*N*
Serotonin	10^{-4}	32
Serotonin	10^{-3}	34
Serotonin	10^{-2}	34
Ringer (serotonine control)	–	33
Dopamine	10^{-4}	38
Dopamine	10^{-3}	37
Dopamine	10^{-2}	36
Ringer (dopamine control)	–	37
Tyramine	10^{-4}	49
Tyramine	10^{-3}	50
Tyramine	10^{-2}	46
Ringer (tyramine control)	–	47
Octopamine	10^{-4}	38
Octopamine	10^{-3}	40
Octopamine	10^{-2}	34
Ringer (octopamine control)	–	41
Epinastine	10^{-2}	36
Epinastine + octopamine	$10^{-2}/10^{-3}$	37
Octopamine	10^{-3}	38
Ringer (epinastine control)	–	38
Mianserin	10^{-2}	42
Mianserin + octopamine	$10^{-2}/10^{-3}$	46
Octopamine	10^{-3}	42
Ringer (mianserin control)	–	42

considered ‘bees with low sensitivity’. Typically, these bees responded to 10% sucrose and/or 30% sucrose or they did not respond at all (GRS 0). Individuals with a GRS greater than 2 were considered ‘bees with higher sensitivity’.

Drug treatment

All bees were tested for their sensitivity to sucrose prior to injection of the substances. Of bees with equal GRS, different groups received different treatments. That way we could exclude effects of prior differences in sensitivity on the outcome of the treatment experiments.

All substances were purchased from Sigma-Aldrich (Munich, Germany). Of each amine (Table 1), we injected 1 µl dissolved in Mobbs ringer solution (270 mmol l^{-1} NaCl, 3.2 mmol l^{-1} KCl, 1.2 mmol l^{-1} CaCl2, 10 mmol l^{-1} MgCl, 10 mmol l^{-1} morpholinopropansulfonic acid, pH 7.4). Solutions were injected into the thorax through a small hole made into the cuticula. A comparison of different treatment methods for octopamine has shown that this method is very effective in increasing brain octopamine titers (Barron et al., 2007). Sensitivity for sucrose was tested again 30 min after injection and the difference between initial sensitivity (GRS) and sensitivity 30 min after injection was calculated for each bee. Comparing the same bees before and after treatment compensated for individual variations in sensitivity. The number of bees tested is shown in Table 1.

Learning assay

Prior to conditioning, bees were tested for their spontaneous response to the conditioned odor carnation. Bees displaying spontaneous proboscis extension to this odor were discarded. For conditioning, a bee was placed in a constant air stream for 8 s. After 2 s, the antennae of the bee were stimulated with 5 ml of an odor air mixture for 3 s, which was delivered by a 20 ml syringe (2 µl odor on a small piece of filter paper) placed in front of the bee. Shortly after the onset of the odor stimulation, the PER was elicited by applying a 30% sucrose solution to the antennae of the bee. Only in one experiment (conditioning foragers with a GRS >2) did we use a 10 % sucrose solution as reward (see Results). When the bee extended her proboscis, she was allowed to drink 1 s from a 1 µl droplet of sucrose solution delivered by the needle of a syringe. After olfactory stimulation, the bee rested in the air stream for 3 s before she was removed from the arena until the next conditioning trial. The inter-trial interval was 5 min.

If the bee did not respond to the sucrose stimulus, she was discarded from further conditioning. If the bee responded with spontaneous PER to the odor stimulus in the first trial she was also discarded. At each of the six conditioning trials we recorded whether the bee displayed a conditioned PER. A learning score was calculated, which ranged from 0 to 5; this score comprises the sum of all conditioned PERs during the acquisition phase (Scheiner et al., 1999; Scheiner et al., 2001a; Scheiner et al., 2001b; Scheiner et al., 2005).

Statistics

For graphic display of learning scores, we calculated median scores and quartiles (SPSS 19.0, IBM, New York, NY, USA), because these scores were not distributed normally (Kolmogorov–Smirnov test; SPSS 19.0). Sensitivity for sucrose was measured as GRS (see above). Changes in sensitivity after drug treatment are shown as the differences between the GRS 30 min after drug treatment and that prior to drug treatment (‘GRS difference’). As gustatory scores were not distributed normally, medians and upper and lower quartiles of GRS differences are displayed. GRS differences were compared between more than two groups using Kruskall–Wallis *H*-tests (SPSS 19.0). Dunn’s test was employed as a *post hoc* test (Graphpad Instat, Graphpad Software Inc., La Jolla, CA, USA). GRSs or learning scores of two groups of bees were compared using Mann–Whitney *U*-tests (SPSS 19.0).

RESULTS

Which age groups learn poorly in honey bees?

A comparison of olfactory and tactile learning performance across different age/behavioral groups suggests that newly emerged bees and foragers with long foraging duration (>15 days) display the poorest learning performance (Fig. 1). Therefore, these groups were selected for our behavioral experiments. Usually, foragers with long foraging durations (>15 days) are older than foragers with short foraging durations (6–13 days), although age *per se* is not important for the learning performance of these behavioral groups (Behrends et al., 2007; Scheiner and Amdam, 2009). For simplicity, we will refer to foragers with long foraging durations as ‘old foragers’.

Which amines can increase sensitivity for sucrose in newly emerged bees?

In this series of experiments, we tested which of the biogenic amines serotonin, dopamine, tyramine and octopamine would increase sensitivity for sucrose in newly emerged bees. Of the four amines tested, only octopamine significantly increased sensitivity for sucrose (two-tailed Kruskal–Wallis *H*-test, H=14.74, $P \leq 0.01$; Fig. 2D). Serotonin, dopamine and tyramine had no effects (Fig. 2A,B,C, respectively). Both octopamine concentrations of 10^{-3} mol l^{-1} and 10^{-2} mol l^{-1} significantly increased sensitivity 30 min after application (Dunn’s multiple comparison test, $P \leq 0.01$) compared with ringer controls.

To test for the specificity of the octopamine effect, we also analyzed the effects of two potent octopamine receptor antagonists, epinastine and mianserine (Roeder et al., 1998; Roeder, 2005).

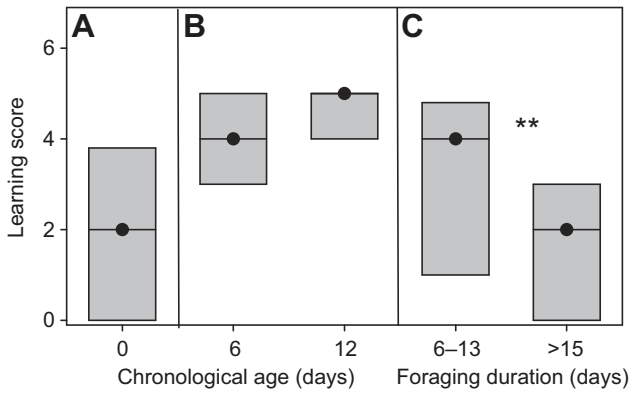

Fig. 1. Learning performance of honey bees changes with age and behavioral role. The *x*-axis displays the age of the bees (A, B) or the foraging duration (C) of foraging bees. The *y*-axis shows median acquisition scores (circles) and 25% and 75% quartiles (lower and upper lines, respectively) of olfactory proboscis extension learning. (A) Learning scores of newly emerged bees are very low (data taken from Behrends and Scheiner, 2009); (B) they gradually increase in hive bees (present study) and (C) decrease with long (>15 days) foraging duration (data taken from Behrends et al., 2007). Asterisks indicate significant differences between the groups of foragers (**$P\leq0.01$; two-tailed Mann–Whitney *U*-test). Direct statistical comparisons between groups of different experiments were inappropriate. Number of bees tested: newly emerged bees, 40; 6-day-old bees, 35; 12-day-old bees, 31; foragers foraging for 6–13 days, 56; and foragers foraging for >15 days, 23.

Treatment significantly affected sensitivity for sucrose (epinastine experiment: $H=19.17$, $P\leq0.001$; mianserine experiment: $H=12.08$, $P\leq0.01$). The increase in sensitivity by action of 10^{-3} mol l^{-1} octopamine compared with ringer controls (Dunn's multiple comparison test, $P\leq0.05$) was reversed by 10^{-2} mol l^{-1} epinastine co-injected with 10^{-3} mol l^{-1} octopamine ($P>0.05$; Fig. 3A) and by 10^{-2} mol l^{-1} mianserine co-injected with octopamine ($P>0.05$; Fig. 3B). These data show that octopamine can specifically increase sensitivity for sucrose in newly emerged honey bees.

Does octopamine application improve associative learning performance in newly emerged bees?

In this experiment, we tested whether octopamine would increase olfactory learning performance in newly emerged bees by increasing their sensitivity for the sucrose reward. At first glance, it seemed that octopamine did not affect learning performance, because learning scores of bees treated with octopamine did not differ from those of ringer controls (Mann–Whitney *U*-test, $Z=0.61$, $P>0.05$; Fig. 4A). However, when bees were grouped according to their sensitivity before treatment, differential effects of octopamine on learning performance became apparent (Fig. 4B,C). Octopamine significantly improved learning performance in bees with low initial sensitivity (GRS≤2; Mann–Whitney *U*-test, $Z=2.11$, $P\leq0.05$; Fig. 4B) but had no effect on bees with higher initial sensitivity (GRS>2; Mann–Whitney *U*-test, $Z=0.05$, $P=0.98$; Fig. 4C). This is probably directly related to the fact that only in bees with low initial sensitivity for sucrose did octopamine increase sensitivity (Fig. 4D). In contrast, octopamine had no effect on the sensitivity of bees with higher initial sensitivity (Fig. 4E). Because the median learning scores and the median GRSs of the octopamine-treated bees with high initial sensitivity were fairly low, we can exclude the possibility that ceiling effects of the data might obscure the octopamine effects.

These findings are the first to demonstrate a causal relationship between sensitivity for sucrose and olfactory learning performance in newly emerged honey bees. Further, they show a differential effect of octopamine on individuals with low and high gustatory sensitivity.

Does octopamine increase sensitivity for sucrose and learning performance in old foragers?

As in newly emerged bees, 10^{-3} mol l^{-1} octopamine significantly increased sensitivity in foragers with long foraging duration

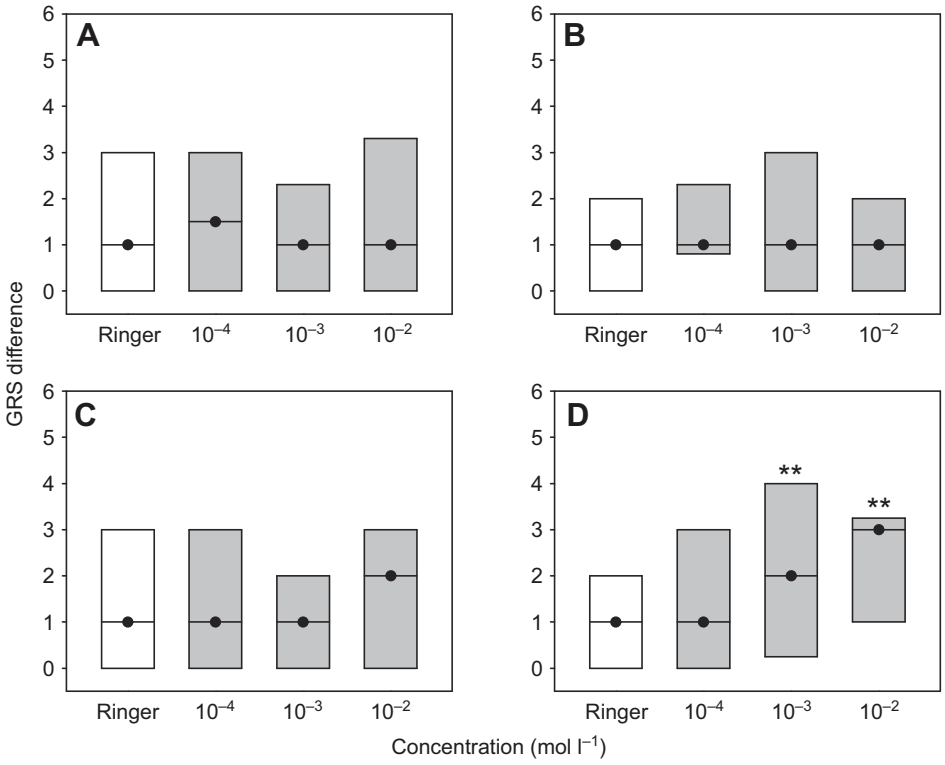

Fig. 2. The action of the biogenic amines serotonin (A), dopamine (B), tyramine (C) and octopamine (D) on sensitivity to sucrose in newly emerged honey bees. Sensitivity was measured as gustatory response scores (GRS, see Materials and methods). The difference in GRS 30 min after drug application compared with the GRS prior to treatment shows the change in sensitivity due to drug treatment. The *x*-axis displays the concentrations of the substances that were injected or the ringer control. The *y*-axis displays the change in GRS 30 min after injection of the substances. Median GRS differences (circles) and quartiles (upper and lower lines) are shown. The numbers of bees tested are shown in Table 1. Asterisks indicate significant differences between treatment groups and ringer controls (**$P\leq0.01$; Dunn's multiple comparison test).

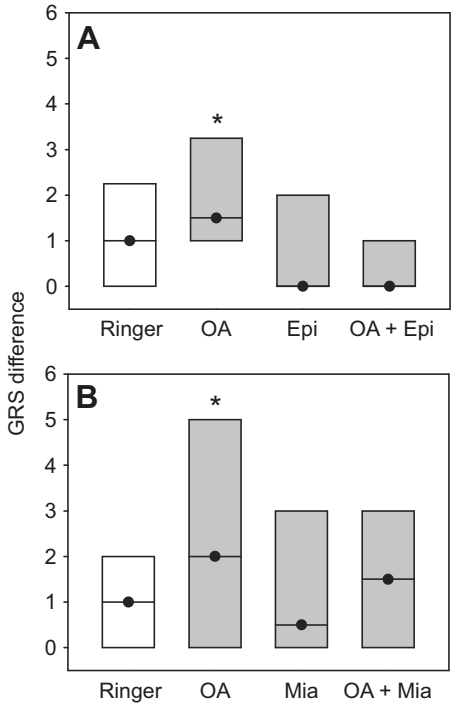

Fig. 3. The action of the biogenic amine octopamine (OA) at a concentration of 10^{-3} mol l^{-1} on sensitivity to sucrose in newly emerged honey bees can be reversed by the octopamine receptor antagonists epinastine (10^{-2} mol l^{-1}; Epi) and mianserine (10^{-2} mol l^{-1}; Mia). The *x*-axis displays the substances that were injected or the ringer control. The *y*-axis displays the change in gustatory response score (GRS) 30 min after injection of the substances compared with the GRS prior to injection. Median GRS differences (circles) and quartiles (upper and lower lines) are shown. The numbers of bees tested are shown in Table 1. Asterisks indicate significant differences between the ringer control and a treatment group (*$P \leq 0.05$; Dunn's multiple comparison test).

(Mann–Whitney *U*-test, Z=1.99, $P \leq 0.05$; Fig. 5A). Olfactory learning scores of all foragers treated with 10^{-3} mol l^{-1} octopamine, however, did not differ from those of foragers treated with ringer solution (Mann–Whitney *U*-test, Z=0.27, P=0.79; Fig. 5B). We next divided the foragers into groups with low sensitivity (GRS ≤2) and high sensitivity (GRS >2) prior to treatment, similar to the grouping of newly emerged bees (Fig. 5C,D, respectively). This time, however, foragers with low initial sensitivity did not differ in their olfactory learning performance from ringer controls (Mann–Whitney *U*-test, Z=0.19, P=0.22; Fig. 5C). In contrast to newly emerged bees with similar low sensitivity, they showed a slightly impaired learning performance. Old foragers with high initial sensitivity that were treated with 10^{-3} mol l^{-1} octopamine also did not differ from ringer controls in their learning scores (Mann–Whitney *U*-test, Z=1.51, P=0.15; Fig. 5D). Sometimes, ceiling effects of the data can obscure the effects of treatments. The old foragers with high initial GRSs that were treated with octopamine (Fig. 5D) had a median learning score of 5, which corresponds to the maximum learning score. Because of the good learning performance of the control group (median of 4) it might have been difficult to detect a significant effect of octopamine treatment on this group. Therefore, we trained a second group of foragers with GRSs >2 that had been treated with ringer or octopamine solution, this time using a 10% sucrose solution as reward. The lower sucrose concentration used as reward slightly slowed down the acquisition rate. Both the ringer group and the octopamine group had a median learning score of 4 and again did not differ significantly in their learning scores (data not shown; Mann–Whitney *U*-test, Z=0.07, P=0.95, N_{ringer}=13, $N_{octopamine}$=16). Our data therefore show that the improvement in learning performance through octopamine that we observed in newly emerged bees could not be replicated in foragers, although octopamine increased sensitivity for sucrose in this age group.

DISCUSSION

Relationship between sensitivity to sucrose and learning performance

Our results show for the first time a causal relationship between sensitivity to sucrose and appetitive learning in newly emerged honey bees. When we increased sensitivity for sucrose in newly emerged bees with low initial sensitivity, olfactory learning performance improved significantly. Most of our earlier experiments on the role of individual sensitivity for sucrose for appetitive learning in honey bees have demonstrated correlations between GRS and learning scores (Scheiner et al., 1999; Scheiner et al., 2001a; Scheiner et al., 2001b; Scheiner et al., 2005). We believe that these correlations are indicative for causal relationships between sensitivity for sucrose and appetitive learning performance, at least in young honey bees, because we did not observe such a causal relationship in old foragers. The latter age group is special in that the impaired learning performance of old foragers is linked to a high sensitivity for sucrose (Behrends et al., 2007; Scheiner and Amdam, 2009; Tolfsen et al., 2011).

Differences in the learning performance of individual young bees reflect differences in their sensory sensitivity. Therefore, individual sensitivity for sucrose can be regarded as a major motivational factor for honey bee appetitive learning. This makes it an excellent predictor of appetitive learning performance. In future experiments requiring a high-throughput learning test, it might therefore suffice to measure the sensitivity for sucrose of individual young bees to make accurate predictions of their appetitive learning behavior.

Despite the large impact of sensitivity for the reward in olfactory PER learning, olfactory sensitivity naturally also plays a role in learning performance, because odor intensity affects learning performance (Bhagavan and Smith, 1996; Wright et al., 2005). As we earlier found a correlation between sensitivity to sucrose and sensitivity to odors (Scheiner et al., 2004), the differential effects of odor intensity and sugar concentration of the reward are difficult to separate. Based on PER learning experiments in which we rewarded bees with different sensitivities with equal subjective rewards, so that their learning performance did not differ significantly (Scheiner et al., 2005), we assume that the impact of sensitivity to odors is of minor importance for appetitive learning compared with that of sensitivity to sucrose.

Octopamine increases sensitivity to sucrose

Of the different biogenic amines tested in this experimental series, only octopamine reliably increased sensitivity to sucrose in newly emerged bees and old foragers. Serotonin, dopamine and tyramine had no effect. The octopamine effect was reversed by co-application of the octopamine receptor antagonists mianserine and epinastine.

Our results are well in line with earlier experiments on the action of octopamine on sensory sensitivity in insects. All of these show that octopamine enhances sensory sensitivity, be it for odors (Pophof, 2002; Spivak et al., 2003), visual stimuli (for a review, see Erber et al., 1993) or gustatory stimuli (Menzel et al., 1988; Mercer and Menzel, 1982; Scheiner et al., 2006).

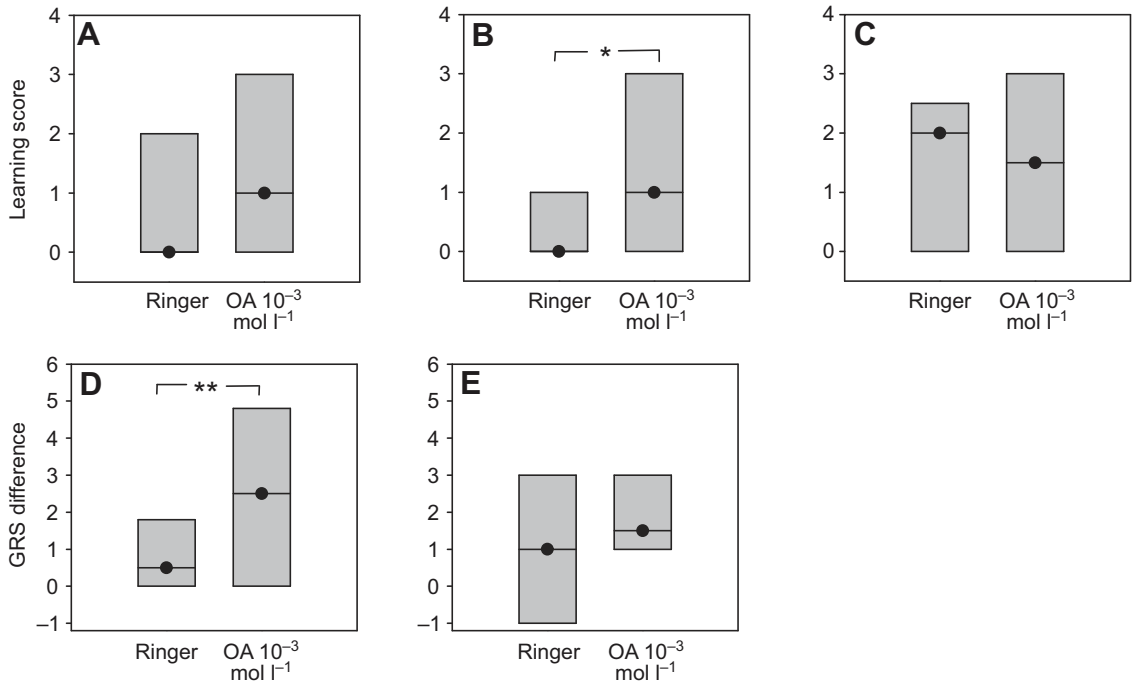

Fig. 4. Octopamine (OA) improves associative learning in newly emerged honey bees with low initial sensitivity. (A) Median olfactory learning scores (circles) and quartiles (upper and lower lines) of all newly emerged bees treated with 10^{-3} mol l^{-1} OA and ringer controls. (B) Learning scores of a subsample of bees from A with low initial sensitivity (GRS ≤2). (C) Learning scores of a subsample of bees from A with high initial sensitivity (GRS >2). (D) Median difference in GRS and quartiles 30 min after OA or ringer treatment of bees shown in B. (E) Median difference in GRS and quartiles 30 min after OA or ringer treatment of bees shown in C. Significant differences between groups are indicated by asterisks (*P≤0.05; **P≤0.01; Mann–Whitney U-test). Number of bees tested: (A) ringer, 49; OA, 55; (B) ringer, 36; OA, 41; and (C) ringer, 13; OA, 14.

Octopamine increases sensitivity for sucrose not only in unresponsive age groups of bees but also in satiated bees. Out of a population of foragers, almost 100% of hungry individuals showed proboscis extension to a 30% sucrose stimulus. When bees were satiated, the number of responding bees dropped sharply. Octopamine injections into the brain *via* the ocellar tract have been shown to restore the response to almost 100% (Menzel et al., 1988). Whether the same or different mechanisms are involved in the modulation of age-dependent sensitivity to sucrose and in the hunger-modulated sensitivity to sucrose needs to be tested experimentally. But octopamine plays a decisive part in both pathways.

We assume that the observed increase in sensitivity was achieved through increased activation of octopamine receptors in the brain of the bee. There are five octopamine receptors in the honey bee brain (Grohmann et al., 2003; Hauser et al., 2006). Whether some or all of these receptors are involved in the regulation of sensitivity for gustatory stimuli is currently unknown, but is under investigation in our laboratory. Downregulation of one of these receptors by RNA interference (Farooqui et al., 2003) led to a decreased learning performance. We assume that this effect was due to a decreased sensitivity for the sucrose reward. The downregulation of gene expression of each octopamine receptor will therefore be an important step in studying the functions of these receptors for sensory sensitivity and learning of honey bees.

Differential effects of octopamine on bees differing in initial sensitivity

Octopamine did not act similarly on all newly emerged bees or old foragers. The effects of this amine were strongly dependent on the initial sensitivity of the treated bees. In newly emerged bees and old foragers with low initial sensitivity, octopamine made bees more sensitive, whereas it had no effect on bees with higher initial sensitivity. Learning performance was only improved in newly emerged bees with low initial sensitivity.

This finding is of great importance for interpreting the actions of biogenic amines or other modulators on the behavior of honey bees and other insects and even vertebrates. It suggests that the individual physiological state, which in honey bees can be estimated by measuring sensitivity to sucrose, is an important determinant for the effectiveness of treatments. Thus we can explain seemingly odd results from a number of studies aiming at modulating honey bee behavior. (1) Pankiw and Page (Pankiw and Page, 2001) found that application of brood pheromone decreased sucrose response thresholds in honey bees, but found the opposite effect of brood pheromone in a later study (Pankiw and Page, 2003). We assume that the sensitivity to sucrose of the bee population tested in the first experiment was lower than that of the second experiment. Brood pheromone might only increase sensory sensitivity in bees with low initial sensitivity. (2) The group of Marla Spivak showed that bees selected for hygienic behavior (they detect, uncap and remove diseased brood quickly) are more sensitive to the odor of diseased brood than unhygienic bees (Masterman et al., 2001). Octopamine application increased sensitivity to the odor of diseased brood in bees of the unhygienic strain but not in bees of the hygienic strain. Further, the octopamine receptor antagonist epinastine reduced olfactory sensitivity in hygienic bees but not in unhygienic bees, which were already quite insensitive (Spivak et al., 2003). We hypothesize that octopamine only led to an increase in olfactory sensitivity in unhygienic bees, because these bees were initially less

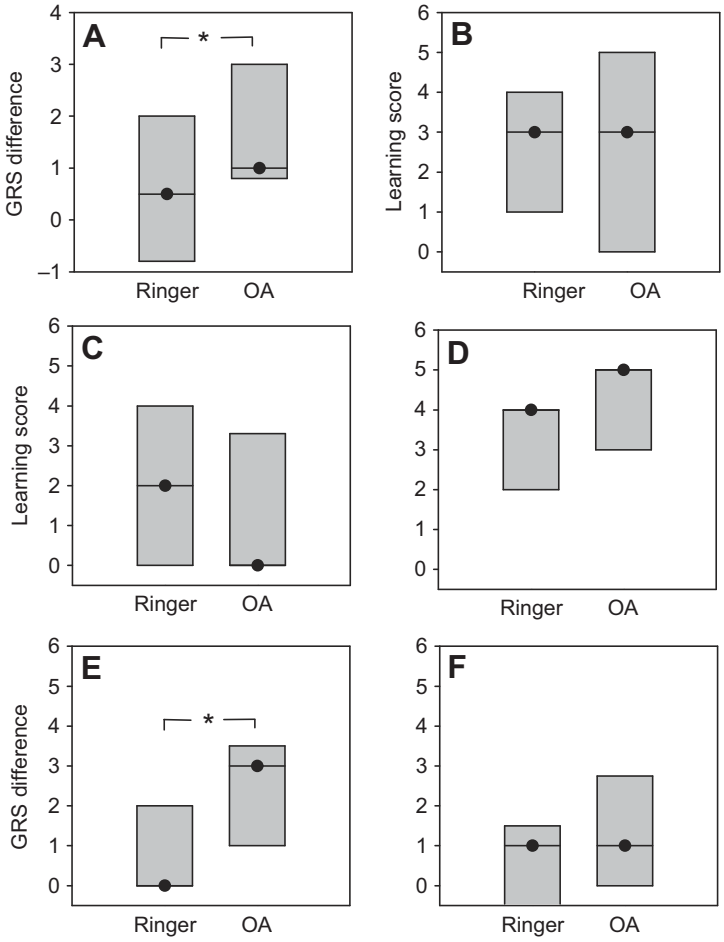

Fig. 5. Octopamine (OA) increases sensitivity to sucrose in old foragers but it does not increase learning scores. (A) Median difference in GRS (circles) and quartiles (upper and lower lines) 30 min after OA or ringer treatment of all old foragers. (B) Median learning scores (circles) and quartiles (upper and lower lines) of all old foragers treated with OA or ringer solution. (C) Learning scores of a subsample of foragers from B with low initial sensitivity (GRS ≤2). (D) Learning scores of a subsample of foragers from B with high initial sensitivity (GRS >2). (E) Median difference in GRS (circles) and quartiles (upper and lower lines) of a subsample of foragers from B with low initial sensitivity (GRS ≤2). (F) Median difference in GRS (circles) and quartiles (upper and lower lines) of a subsample of foragers from B with high initial sensitivity (GRS >2). Asterisks indicate significant differences between groups (*P≤0.05; Mann–Whitney U-test). Number of bees tested: (A) ringer, 20; OA, 22; (B) ringer, 31; OA, 27; (C) ringer, 16; OA, 12; and (D) ringer, 15; OA, 15.

sensitive than the hygienic bees. Accordingly, epinastine might only have affected olfactory sensitivity in highly responsive bees. We suggest that in future experiments analyzing the function of amines and pheromones in sensory sensitivity or learning behavior, individual sensitivity should be tested prior to treatment. That way, effects of drugs might become visible earlier and the sample size needed to detect a small effect can be decreased.

Age-dependent effects of octopamine on learning performance

Octopamine increased sensitivity to sucrose in insensitive newly emerged bees and old foragers alike. But to our surprise, octopamine only improved learning performance in newly emerged bees. Insensitive old foragers even displayed a slight decrease in learning scores after octopamine treatment. This finding is particularly interesting because it suggests that the cause for the poor learning performance of old foragers is different from that of newly emerged bees. In fact, earlier experiments of our group have shown that newly emerged bees learn particularly poorly because they are extremely insensitive to low sugar concentrations (Behrends and Scheiner, 2009). Old foragers, in contrast, shared the same sensitivity for sucrose as same-aged nurse bees. Despite their similar sensitivity, old foragers displayed a significantly poorer learning performance than same-aged hive bees (Behrends et al., 2007; Scheiner and Amdam, 2009; Tolfsen et al., 2011). This suggests that the poor learning performance of old foragers is related to an age-related impairment of structures or pathways involved in associative learning rather than to a low sensitivity for the reward. Seehuus et al. (Seehuus et al., 2006) demonstrated high levels of oxidative carbonylation in the brains of old foragers, suggesting that damage of brain neuropiles could be responsible for the observed learning deficits.

Another reason why octopamine did not improve acquisition, although it made the old foragers more sensitive to the sucrose reward, could be related to the fact that these bees have higher intrinsic octopamine titers than newly emerged bees (Harris and Woodring, 1992). In insensitive old foragers, octopamine application could reduce learning performance by binding to other amine receptors (i.e. tyramine receptors), which partly act in an antagonistic way to octopamine receptors by decreasing cAMP levels in the cell (Blenau et al., 2000; Roeder, 2005).

Some earlier studies on the role of octopamine for behavior used reserpine to deplete biogenic amines from the brains of bees before applying octopamine. In those experiments, octopamine specifically acted on acquisition but not on retrieval of olfactory information (Menzel et al., 1999). In our experiments, we added octopamine to normal intrinsic transmitter levels. Our experiments directly support the assumption made by Menzel et al. (Menzel et al., 1999) that this additional octopamine leads to a general arousing effect, whereas their effects of octopamine on reserpinised bees was rather specific. We assume that the old foragers in our experiment had reduced capabilities for acquiring new information, so that a general arousing effect of octopamine could not improve their learning performance, despite increasing their sensitivity and thus their motivation to learn. In newly emerged bees, in contrast, the increased level of arousal, which led to a higher sensitivity for sucrose, was sufficient to induce faster acquisition.

LIST OF ABBREVIATIONS

GRS gustatory response score
PER proboscis extension response

ACKNOWLEDGEMENTS

We thank Benedict Polazcek for his advice on beekeeping and Anna Toteva for assistance with the behavioral experiments.

FUNDING

This work was supported by grants from the Deutsche Forschungsgemeinschaft [SCHE 1573/1-1 and SCHE 1573/2-1].

REFERENCES

Barron, A. B., Maleszka, J., Vander Meer, R. K., Robinson, G. E. and Maleszka, R. (2007). Comparing injection, feeding and topical application methods for treatment of honeybees with octopamine. *J. Insect Physiol.* **53**, 187-194.

Behrends, A. and Scheiner, R. (2009). Evidence for associative learning in newly emerged honey bees (*Apis mellifera*). *Anim. Cogn.* **12**, 249-255.

Behrends, A., Scheiner, R., Baker, N. and Amdam, G. V. (2007). Cognitive aging is linked to social role in honey bees (*Apis mellifera*). *Exp. Geront.* **42**, 1146-1153.

Ben-Shahar, Y. and Robinson, G. E. (2001). Satiation differentially affects performance in a learning assay by nurse and foragers honey bees. *J. Comp. Physiol. A* **187**, 891-899.

Bhagavan, S. and Smith, B. H. (1996). Olfactory conditioning in the honeybee, *Apis mellifera*: the effects of odor intensity. *Physiol. Behav.* **61**, 107-117.

Bitterman, M. E., Menzel, R., Fietz, A. and Schäfer, S. (1983). Classical conditioning of proboscis extension in honeybees (*Apis mellifera*). *J. Comp. Psychol.* **97**, 107-119.

Blenau, W., Balfanz, S. and Baumann, A. (2000). Amtyr1: characterization of a gene from honeybee (*Apis mellifera*) brain encoding a functional tyramine receptor. *J. Neurochem.* **74**, 900-908.

Braun G. and Bicker, G. (1992). Habituation of an appetitive reflex in the honeybee. *J. Neurophysiol.* **67**, 588-598.

Erber, J., Kloppenburg, P. and Scheidler, A. (1993). Neuromodulation by serotonin and octopamine in the honeybee: behaviour, neuroanatomy and electrophysiology. *Experientia* **49**, 1073-1083.

Farooqui, T., Robinson, K. and Vaessin, H. (2003). Modulation of early olfactory processing by an octopaminergic reinforcement pathway in the honeybee. *J. Neurosci.* **23**, 5370-5380.

Friedrich, A., Thomas, U. and Müller, U. (2004). Learning at different satiatio nlevels reveals parallel functions for the cAMP-protein kinase A cascade in formation of long-term memory. *J. Neurosci.* **24**, 4460-4468.

Fussnecker, B. L., Smith, B. H. and Mustard, J. A. (2006). Octopamine and tyramine influence the behavioral profile of locomotor activity in the honey bee (*Apis mellifera*). *J. Insect Physiol.* **52**, 1083-1092.

Giurfa, M. (2007). Behavioral and neural analysis of associative learning in the honeybee: a taste from the magic well. *J. Comp. Physiol. A* **193**, 801-824.

Grohmann, L., Blenau, W., Erber, J., Ebert, P. R., Strünker, T. and Baumann, A. (2003). Molecular and functional characterization of an octopamine receptor from honeybee (*Apis mellifera*) brain. *J. Neurochem.* **86**, 725-735.

Hammer M. (1993). An identified neuron mediates the unconditioned stimulus in associative olfactory learning in honeybees. *Nature* **366**, 59-63.

Hammer M. (1997). The neural basis of associative reward learning in honeybees. *Trends Neurosci.* **20**, 245-252.

Harris, J. W. and Woodring, J. (1992). Effects of stress, age, season, and source colony on levels of octopamine, dopamine, and serotonin in the honey bee (*Apis mellifera* L.) brain. *J. Insect Physiol.* **38**, 29-35.

Hauser, F., Cazzamali, G., Williamson, M., Blenau, W. and Grimmelikhuijzen, C. J. P. (2006). A review of neurohormone GPCRs present in the fruitfly *Drosophila melanogaster* and the honey bee *Apis mellifera. Progr. Neurobiol.* **80**, 1-19.

Kutsukake, M., Komatsu, A. and Yamamoto, D. (2000). A tyramine receptor gene mutation causes a defective olfactory behavior in *Drosophila melanogaster. Gene* **245**, 31-42.

Kuwabara, M. (1957). Building the conditioned reflex of the Pavlov-type in the honeybee, *Apis mellifera. J. Fac. Sci. Hokkaido Univ. Zool.* **13**, 458-464 (translated from German).

MacMillan, C. S. and Mercer, A. R. (1987). An investigation of the role of dopamine in the antennal lobes of the honeybee, *Apis mellifera. J. Comp. Physiol. A* **160**, 359-366.

Masterman, R., Ross, R., Mesce, K. and Spivak, M. (2001). Olfactory and al response thresholds to odors of diseased brood differ between hygienic and non-hygienic honey bees (*Apis mellifera* L.). *J. Comp. Physiol. A* **187**, 441-452.

Menzel, R. and Müller, U. (1996). Learning and memory in honeybees: from behavior to neural substrates. *Rev. Neurosci.* **19**, 379-404.

Menzel, R., Michelsen, B., Rüffer, P. and Sugawa, M. (1988). Neuropharmacology of learning and memory in honey bees. In *Modulation of Synaptic Transmission and Plasticity in Nervous Systems*, Vol. H19 (ed. G. Hertting and H.-C. Spatz), pp. 333-350. Berlin, Heidelberg: Springer.

Menzel, R., Wittstock, S. and Sugawa, M. (1990). Chemical codes of learning and memory in honey bees. In *The Biology of Memory*, Vol. 23 (ed. L. Squire and K. Lindenlaub), pp. 335-360. Stuttgart: Schattauer.

Menzel, R., Heyne, A., Kinzel, K., Gerber, B. and Fiala, A. (1999). Pharmacological dissociation between the reinforcing, sensitising and response-releasing functions of reward in honeybee classical conditioning. *Behav. Neurosci.* **113**, 744-754.

Mercer A. R. and Menzel, R. (1982). The effects of biogenic amines on conditioned and unconditioned responses to olfactory stimuli in the honeybee A*pis mellifera. J. Comp. Physiol. A* **145**, 363-368.

Page, R. E., Jr and Fondrk, K. (1995). The effects of colony-level selection on the social organization of honey bee (*Apis mellifera* L.) colonies: colony-level components of pollen hoarding. *Behav. Ecol. Sociobiol.* **36**, 135-144.

Page, R. E., Jr, Erber, J. and Fondrk, M. K. (1998). The effect of genotype on response thresholds to sucrose and foraging behavior of honey bees (*Apis mellifera* L.). *J. Comp. Physiol. A* **182**, 489-500.

Pankiw, T. and Page, R. E., Jr (1999). The effect of genotype, age, sex, and caste on response thresholds to sucrose and foraging behavior of honey bees (*Apis mellifera* L.). *J. Comp. Physiol.* A **185**, 207-213.

Pankiw, T. and Page, R. E., Jr (2001). Brood pheromone modulates honeybee (*Apis mellifera* L.) sucrose response thresholds. *Behav. Ecol. Sociobiol.* **49**, 206-213.

Pankiw, T. and Page, R. E., Jr (2003). Effect of pheromones, hormones, and handling on sucrose response thresholds of honey bees (*Apis mellifera* L.). *J. Comp. Physiol. A* **189**, 675-684.

Pophof, B. (2002). Octopamine enhances moth olfactory responses to pheromones, but not those to general odorants. *J. Comp. Physiol. A* **188**, 659-662.

Roeder, T. (2005). Tyramine and octopamine: ruling behavior and metabolism. *Annu. Rev. Entomol.* **50**, 447-477.

Roeder, T., Degen, J. and Gewecke, M. (1998). Epinastine, a highly specific antagonist of insect neuronal octopamine receptors. *Eur. J. Pharmacol.* **349**, 171-177.

Sandoz, J. C., Roger, B. and Pham-Delègue, M. (1995). Olfactory learning and memory in the honeybee: comparison of different classical conditioning procedures of the proboscis extension response. *C. R. Acad. Sci. III* **318**, 749-755.

Sandoz, J. C., Hammer, M. and Menzel, R. (2002). Side-specificity of olfactory learning in the honeybee: US input side. *Learn. Mem.* **9**, 337-348.

Scheiner, R. and Amdam, G. V. (2009). Impaired tactile learning is related to social role in honey bees. *J. Exp. Biol.* **212**, 994-1002.

Scheiner, R. and Erber, J. (2009). Sensory thresholds, learning and the division of foraging labor in the honey bee. In *Organization of Insect Societies* (ed. J. Gadau and J. H. Fewell), pp. 335-356. Cambridge, MA: Harvard University Press.

Scheiner, R., Erber, J. and Page, R. E., Jr (1999). Tactile learning and the individual evaluation of the reward in honey bees (*Apis mellifera* L.). *J. Comp. Physiol. A* **185**, 1-10.

Scheiner, R., Page, R. E., Jr and Erber, J. (2001a). Responsiveness to sucrose affects tactile and olfactory learning in preforaging honey bees of two genetic strains. *Behav. Brain Res.* **120**, 67-73.

Scheiner, R., Page, R. E., Jr and Erber, J. (2001b). The effects of genotype, foraging role and sucrose perception on the tactile learning performance of honey bees (*Apis mellifera* L.). *Neurobiol. Learn. Mem.* **76**, 138-150.

Scheiner, R., Plückhahn, S., Öney, B., Blenau, W. and Erber, J. (2002). Behavioural pharmacology of octopamine, tyramine and dopamine in honey bees. *Behav. Brain Res.* **136**, 545-553.

Scheiner, R., Barnert, M. and Erber, J. (2003). Variation in water and sucrose responsiveness during the foraging season affects proboscis extension learning in honey bees. *Apidologie* **34**, 67-72.

Scheiner, R., Page, R. E. and Erber, J. (2004). Sucrose responsiveness and behavioral plasticity in honey bees (*Apis mellifera*). *Apidol.* **35**, 133-142.

Scheiner, R., Kuritz-Kaiser, A., Menzel, R. and Erber, J. (2005). Sensory responsiveness and the effects of equal subjective rewards on tactile learning and memory of honeybees. *Learn. Mem.* **12**, 626-635.

Scheiner, R., Blenau, W. and Baumann, A. (2006). Aminergic control and modulation of honeybee behaviour. *Curr. Neuropharmacol.* **4**, 259-276.

Schroll, C., Riemensperger, T., Bucher, D., Ehmer, J., Völler, T., Erbauth, K., Gerber, B., Hendel, T., Nagel, G., Buchner, E. and Fiala, A. (2006). Light-induced activation of distinct modulatory neurons triggers appetitive or aversive learning in *Drosophila* larvae. *Curr. Biol.* **16**, 1741-1747.

Schwärzel, M., Monastirioti, M., Scholz, H., Friggi-Grelin, F., Birman, S. and Heisenberg, M. (2003). Dopamine and octopamine differentiate between aversive and appetitive olfactory memories in *Drosophila. J. Neurosci.* **23**, 10495-10502.

Seehuus, S. C., Krekling, T. and Amdam, G. V. (2006). Cellular senescence in honey bee brain is largely independent of chronological age. *Exp. Gerontol.* **41**, 1117-1125.

Spivak, M., Masterman, R., Ross, R. and Mesce, K. A. (2003). Hygienic behavior in the honey bee (*Apis mellifera* L.) and the modulatory role of octopamine. *J. Neurobiol.* **55**, 341-354.

Tolfsen, C. C., Baker, N., Kreibich, C. and Amdam, G. V. (2011). Flight restriction prevents associative learning deficits but not changes in brain protein-adduct formation during honeybee ageing. *J. Exp. Biol.* **214**, 1322-1332.

Unoki S., Matsumoto, Y. and Mizunami, M. (2005). Participation of octopaminergic reward system and dopaminergic punishment system in insect olfactory learning revealed by pharmacological study. *Eur. J. Neurosci.* **22**, 1409-1416.

Vergoz, V., Roussel, E., Sandoz, J. C. and Giurfa, M. (2007). Aversive learning in honeybees revealed by the olfactory conditioning of the sting extension reflex. *PLoS ONE* **2**, e288.

Wright, G. A., Thomson, M. G. and Smith, B. H. (2005). Odour concentration affects odour identity in honeybees. *Proc. R. Soc. Lond. B* **272**, 2417-2422.

Wright, G. A., Mustard, J. A., Kottcamp, S. M. and Smith, B. H. (2007). Olfactory memory formation and the influence of reward pathway during appetitive learning by honey bees. *J. Exp. Biol.* **210**, 4024-4033.