

RESEARCH ARTICLE

Learning, gustatory responsiveness and tyramine differences across nurse and forager honeybees

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ABSTRACT

Honeybees are well known for their complex division of labor. Each bee sequentially performs a series of social tasks during its life. The changes in social task performance are linked to gross differences in behavior and physiology. We tested whether honeybees performing different social tasks (nursing versus foraging) would differ in their gustatory responsiveness and associative learning behavior in addition to their daily tasks in the colony. Further, we investigated the role of the biogenic amine tyramine and its receptors in the behavior of nurse bees and foragers. Tyramine is an important insect neurotransmitter, which has long been neglected in behavioral studies as it was believed to only act as the metabolic precursor of the better-known amine octopamine. With the increasing number of characterized tyramine receptors in diverse insects, we need to understand the functions of tyramine on its own account. Our findings suggest an important role for tyramine and its two receptors in regulating honeybee gustatory responsiveness, social organization and learning behavior. Foragers, which were more responsive to gustatory stimuli than nurse bees and performed better in appetitive learning, also differed from nurse bees in their tyramine brain titers and in the mRNA expression of a tyramine receptor in the brain. Pharmacological activation of tyramine receptors increased gustatory responsiveness of nurse bees and foragers and improved appetitive learning in nurse bees. These data suggest that a large part of the behavioral differences between honeybees may be directly linked to tyramine signaling in the brain.

KEY WORDS: Biogenic amines, Nurse bee, *Apis mellifera*, PER, Proboscis extension response

INTRODUCTION

Honeybees are famous for their intricate division of labor. Each bee sequentially performs a series of social tasks during its life (for a review, see Johnson, 2010). The most pronounced changes in individual behavior occur when nurse bees transition to foraging behavior. While nurse bees stay inside the hive and provide the brood with food, foragers leave the colony to collect pollen and nectar (Johnson, 2010). Nurse bees are typically between 1 and 2 weeks of age, while bees on average start foraging at 3 weeks of age. While nurse bees have active brood food glands, high levels of the egg-yolk precursor protein vitellogenin and low titers of the

developmental hormone juvenile hormone (Hunt et al., 2007), foragers have deteriorated brood food glands, low vitellogenin titers and high amounts of juvenile hormone. In addition, foragers have a strong circadian activity rhythm, while nurse bees are active around the clock (Shemesh et al., 2010).

According to a widely accepted hypothesis, the complex social organization in honeybees is based on individual differences in sensory response thresholds (Robinson, 1992; Ament et al., 2010). Indeed, bees performing different foraging tasks (i.e. pollen foragers versus nectar foragers) have been shown to differ in their response thresholds for gustatory and visual stimuli (Page et al., 1998; Scheiner et al., 2001a,b), and bees performing tasks in the hive were shown to be less responsive to visual stimuli than foragers (Thamm and Scheiner, 2014). The biogenic amines serotonin, dopamine, octopamine and tyramine are prime candidates for regulating division of labor through modulation of sensory response thresholds (Scheiner et al., 2006). Although serotonin, dopamine and octopamine have been studied in some detail with respect to their role in modulating behavioral responses (Blenau and Baumann, 2001, 2015; Blenau and Thamm, 2011; Ellen and Mercer, 2012; Giurfa, 2006; Mercer, 2008; Scheiner et al., 2006; Thamm et al., 2010), tyramine has rarely been studied. It was long believed to be simply a metabolic precursor of the well-known neurotransmitter and neurohormone octopamine. In the last few years, however, more and more specific tyramine receptors have been characterized in diverse insect species (Cazzamali et al., 2005; Huang et al., 2009; Bayliss et al., 2013) and evidence is accumulating that tyramine is released and acts independently of octopamine (Lange, 2009). The honeybee genome contains sequences for two tyramine receptors (Cazzamali et al., 2005; Hauser et al., 2006). One of these tyramine receptors (AmTYR1) decreases intracellular cAMP concentrations ($[cAMP]_i$) when activated (Beggs et al., 2011; Blenau et al., 2000; Mustard et al., 2005), while the second tyramine receptor (AmTYR2) increases $[cAMP]_i$ after activation (Reim et al., 2017). Interestingly, this receptor acts similarly to four of the five honeybee octopamine receptors (Balfanz et al., 2014), which all increase $[cAMP]_i$ after activation. The differential effects on $[cAMP]_i$ of the two tyramine receptors and most of the honeybee octopamine receptors might explain why in some situations, octopamine acts similarly to tyramine, such as in assays involving gustatory responsiveness, in which octopamine and tyramine both increased responsiveness (Scheiner et al., 2002) or aversive learning, in which both octopamine and tyramine can reduce learning performance, although the tyramine effect was very weak (Agarwal et al., 2011). In experiments investigating honeybee foraging behaviour, both tyramine and octopamine showed the trend to induce foragers to collect more dilute nectar or water (Giray et al., 2007), which was probably related to the increased gustatory responsiveness of the bees due to these amines (Scheiner et al., 2002). In contrast, in other experiments investigating phototaxis of honeybee foragers,

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tyramine and octopamine had opposite effects (Scheiner et al., 2014b).

Here we investigate the function of tyramine and its receptors in gustatory responsiveness, learning performance and division of labor in honeybees.

MATERIALS AND METHODS

Bees

Nurse bees and foragers (*Apis mellifera carnica* Pollman 1879) were randomly obtained from a typical honeybee colony comprising approximately 40,000 honeybees. Bees used for determining tyramine brain titer were sampled from hives maintained at Macquarie University Sydney, Australia. Honeybees for behavioral analyses were kept on the grounds of the University of Potsdam, Germany. Tyramine receptor gene expression was measured in honeybees located at the University of Würzburg, Germany.

Nurse bees were sampled from brood frames containing open brood. Only bees poking their head into an open brood cell for at least 10 s were considered nurse bees. Foragers were collected when returning to their colony. To ensure that we only tested foragers and not bees performing observation flights or defecation flights, we only selected foragers with large pollen loads (Thamm and Scheiner, 2014).

To obtain nurse-aged bees for behavioral pharmacology, frames with capped brood were kept in an incubator maintained at 34°C and 65% humidity until the bees emerged. Newly emerged bees received a paint mark at their thorax and were restored to their colony. After 1 week, when most of these bees performed nursing tasks, bees were individually retrieved from the colony (Thamm and Scheiner, 2014).

For the behavioral tests, each bee was individually immobilized on ice and mounted in a small holder. Behavioral tests started 1 h after fixing the bees (Scheiner et al., 2014b). During this time, the bee rested in a humidified chamber.

Gustatory responsiveness

For determining gustatory responsiveness, each bee was sequentially stimulated by presenting a series of sucrose concentrations (0.1, 0.3, 1, 3, 10 and 30% w/v) to her antennae (for details, see Scheiner et al., 2013, 2014a). The sum of proboscis extension responses (PER) to the stimulations with seven different sucrose concentrations constitutes the gustatory response score (GRS) of a bee, which is an excellent measure of its gustatory responsiveness (Scheiner et al., 2003, 2004, 2014a). To evaluate the effect of neuroactive substances, changes in GRS 30 min after application compared with GRS prior to treatment were calculated and compared between groups.

Olfactory learning

Each bee responding to 30% sucrose was conditioned to the odor citral with six learning trials. First, spontaneous PER to the conditioned odor was tested. Bees showing a spontaneous response were excluded from further testing. Next, bees were trained to citral odor (2 µl odor on a piece of filter paper inserted into a 10 ml syringe). Each bee was placed in a constant air stream. During each trial, 5 ml of an odor/air mixture were delivered manually to the antennae of the bee. The bee experienced the odor for 1 s, before the PER was elicited by touching an antenna with a 30% sucrose droplet. As soon as the bee extended its proboscis, it was fed with a small droplet of sucrose solution for 1 s. The odor was removed approximately 0.5 s after the onset of sucrose feeding, so that

conditioned odor and sucrose reward overlapped in time. The inter-trial interval was 5 min. During each conditioning trial, we recorded whether the bee displayed the PER before this response was elicited by applying sucrose to its antennae. The sum of conditioned responses during the training session constituted the acquisition score of the bee (Scheiner et al., 1999).

Behavioral pharmacology

Bees were individually treated with different concentrations of tyramine or its receptor antagonist yohimbine dissolved in phosphate buffered saline (PBS; 140 mmol l⁻¹ NaCl, 2.6 mmol l⁻¹ KCl, 8.1 mmol l⁻¹ Na₂HPO₄, 1.5 mmol l⁻¹ KH₂PO₄, pH 7.4). Each bee was punctured in the middle of its thorax with a thin needle, and 1 µl of PBS solution containing tyramine, yohimbine or both was placed on top of the small whole. After approximately 5 min, the droplet disappeared into the hemolymph. This method had been applied successfully in several earlier experiments to study effects of biogenic amines on honeybee behavior (Pribbenow and Erber, 1996; Robinson et al., 1999; Scheiner et al., 2002), and allowed a high throughput of bees, while elevating tyramine brain titers significantly. Gustatory responsiveness or learning performance was measured again 30 min after injection only in bees without any visible droplet of solution on their thorax.

Quantification of mRNA

Single frozen brains were homogenized in 750 µl of Isol-RNA lysis reagent (5PRIME, Hilden, Germany) and afterwards 150 µl of chloroform was added. After phase separation, the aqueous phase was transferred to 900 µl ethanol (75%). Subsequently, the peqGOLD Total RNA Kit (Peqlab, Erlangen, Germany) was used to purify RNA following the standard protocol including a DNase I digestion step. From each bee, 750 µg of total brain RNA was transcribed using the QuantiTect[®] Reverse Transcription Kit (Qiagen, Hilden, Germany). Five microliters of each cDNA were run in triplicate in a quantitative real-time PCR on a Rotor-Gene Q (Qiagen) with the following protocol: 60°C for 1 min, 95°C for 5 min, and 45 cycles at 95°C for 20 s and 60°C for 1 min. Each reaction (25 µl) contains each primer (0.25 µmol l⁻¹), TaqMan[®] probes (0.1 µmol l⁻¹) and Rotor-Gene Multiplex PCR Master Mix (Qiagen). Sequences of primers and TaqMan probes are given in Table 1. Relative expression to *AmEF1α* (Reim et al., 2013) with the $\Delta\Delta C_t$ method was determined using Rotor-Gene Q software (Qiagen, Chatsworth, CA, USA).

Quantification of tyramine and octopamine brain titers

For quantifying tyramine titers in nurse bees and foragers, individual animals were immediately frozen in liquid nitrogen.

Table 1. DNA oligos used for quantitative real-time PCR

Gene	Oligo name	Oligo sequence
<i>AmEF1α</i>	AmEF1α_qF	GAACATTCTGTGAAAGAGTTGAGGC
	AmEF1α_qR	TTTAAAGGTGACACTCTTAATGACGC
	AmEF1α_TM	6FAM-ACCGAGGAGAATCCGAAGAGCA TCAA-BBQ
<i>Amtyr1</i>	AmTyr1_F	AGCCGACCCGAGGTCCAGATAG
	AmTyr1_R	CCCATTATCACGCCAATGTCC
	AmTyr1_TM	YAK-AACGAGATCCTCTGCCTCTCCTCG ATGAA-BBQ
<i>Amtyr2</i>	AmTyr2_F	GTTACTAATTGTTTCGTGTCCAGCTT
	AmTyr2_R	GCAGTACAGAGAAGAATGTCCGAGG
	AmTyr2_TM	YAK-AGGTACCACCTGTGAGCTGTAAACA GCA-BBQ

For testing whether injection with tyramine into the thorax would lead to elevated tyramine or octopamine titers in the brain, individuals were treated as for measuring tyramine effects on gustatory responsiveness of learning. The heads were removed and lyophilized at -65°C and 320 mTorr for 50 min to remove some water content. Brains were dissected from the head capsule over dry ice while frozen. Dissected brains were stored at -80°C until further processing.

To extract biogenic amines, frozen brains were first centrifuged at 15 g for 2 min at 4°C to begin mechanical disruption of tissue. Brains were then homogenized by sonication in 100 μl of 0.2 mol l^{-1} perchloric acid containing $10\text{ pg } \mu\text{l}^{-1}$ dihydroxybenzylamine. Homogenized brains were incubated on ice in darkness for 20 min, before centrifugation at 15 g for 15 min to pellet cell fragments. The supernatant was collected and 10 μl of the supernatant of each sample was analyzed with HPLC. Content of biogenic amines in the extractant from brain tissue was quantified using an Agilent 1200 Series HPLC system (Agilent Technologies, Santa Clara, CA, USA) with an ESA Coulechem III electrochemical detector connected to an ESA 5011A dual-electrode analytical cell (ESA, Chelmsford, MA, USA). Samples were separated across a 100 mm Hypersil 5 μm octadecylsilane packaged column (Thermo Fisher Scientific, Waltham, MA, USA). Biogenic amine amounts were quantified relative to known amounts of biogenic amines (Søvik et al., 2013; Scheiner et al., 2014b).

Statistics

Biogenic amine brain titers and tyramine receptor gene expression were compared between different groups using two-tailed *t*-tests, because data were distributed normally. As gustatory response scores and acquisition scores were not distributed normally, we applied Mann–Whitney *U*-tests for comparison of two groups and Kruskal–Wallis *H*-tests for comparison of more than two groups followed by Dunn's *post hoc* tests. For display of acquisition curves, the percentage of bees showing the conditioned response was calculated. Learning curves were compared using generalized estimating equations (logistic regression), because data followed a bimodal distribution (Matsumoto et al., 2012). We separately

analyzed the effects of learning trial and behavioral group on learning performance. All tests were two-tailed. Statistics were performed with SPSS 22 (IBM, Armonk, NY, USA).

RESULTS

Social role and behavioral differences of nurse bees and foragers correlate with different tyramine titers and tyramine receptor gene expression in the brain

We asked whether the different social roles of nurse bees (Fig. 1A) and foragers (Fig. 1B) would be related to a differential tyramine signaling, and measured tyramine titers and tyramine receptor gene expression in the brains of both behavioral groups. Intriguingly, foragers displayed significantly higher tyramine levels in their brains than did nurse bees ($t=2.40$, $P<0.01$, $n_{\text{nurse}}=15$, $n_{\text{forager}}=28$; Fig. 1C). Foragers had significantly lower mRNA levels of the tyramine receptor gene *AmTyr2* in their brains than did nurse bees ($t=2.79$, $P<0.05$, $n_{\text{nurse}}=8$, $n_{\text{forager}}=8$; Fig. 1D). In contrast, mRNA levels of the tyramine receptor gene *AmTyr1* did not differ between nurse bees and foragers ($t=1.54$, $P>0.05$, $n_{\text{nurse}}=8$, $n_{\text{forager}}=8$; Fig. 1D). These findings suggest that in honeybees, social role is at least partly related to a differential tyramine signaling.

We next asked whether nurse bees and foragers differed in their gustatory responsiveness, an important behavioral indicator (for review, see Scheiner et al., 2004). Foragers were significantly more responsive to gustatory stimuli than nurse bees ($Z=3.55$, $P<0.001$, $n_{\text{nurse}}=29$, $n_{\text{forager}}=31$; Fig. 2A), demonstrating a relationship between social organization and nutrition-related responsiveness.

Individual gustatory responsiveness is an excellent predictor of appetitive learning ability in honeybees (Scheiner et al., 2001a,b, 2004). We therefore hypothesized that the differences in GRS of nurse bees and foragers should lead to respective differences in their appetitive proboscis extension learning performance. Indeed, foragers performed better in appetitive olfactory learning than nurse bees and consequently reached higher acquisition scores ($Z=2.00$, $P<0.05$, $n_{\text{nurse}}=29$, $n_{\text{forager}}=31$; Fig. 2B). During the course of acquisition, the number of nurse bees and foragers displaying the conditioned PER increased significantly in both nurse bees and foragers (logistic regression, effect of trial: $\chi^2_{4}=12.05$, $P<0.05$).

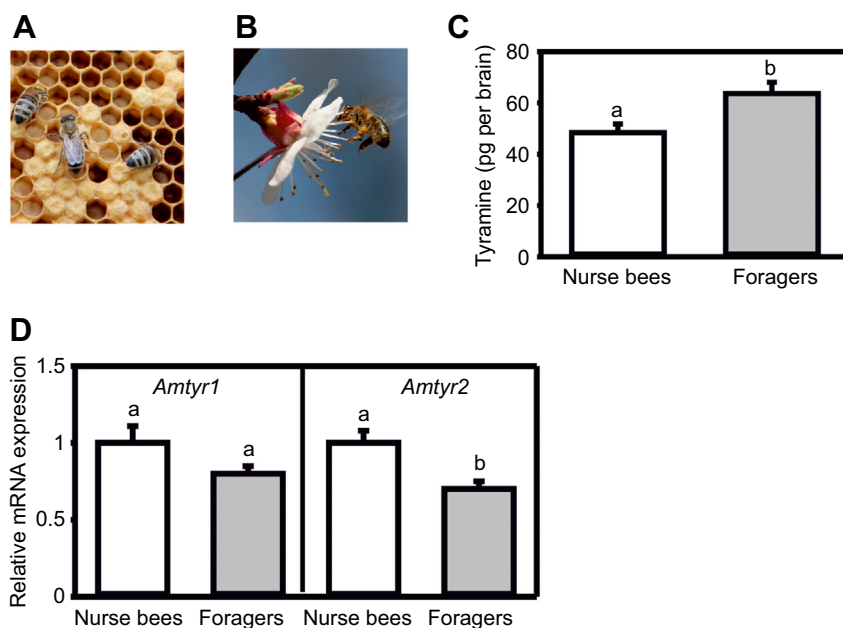


Fig. 1. Differential tyramine signaling in nurse bees and foragers. (A) Nurse bees of *Apis mellifera*. (B) Forager bee of *Apis mellifera*. (C) Tyramine brain titers of nurse bees and foragers. Mean \pm s.e.m titers are displayed. Foragers have significantly higher tyramine brain titers than nurse bees ($n_{\text{forager}}=28$, $n_{\text{nurse}}=15$). (D) Receptor mRNA levels of *Amtyr1* and *Amtyr2* in the brains of nurse bees and foragers. Relative expression levels and standard errors are displayed. Nurse bees display a significantly higher relative mRNA expression in the brain compared with foragers ($n_{\text{forager}}=8$, $n_{\text{nurse}}=8$). Significant differences between groups are indicated by different letters ($P<0.05$). For details of tests, see Results.

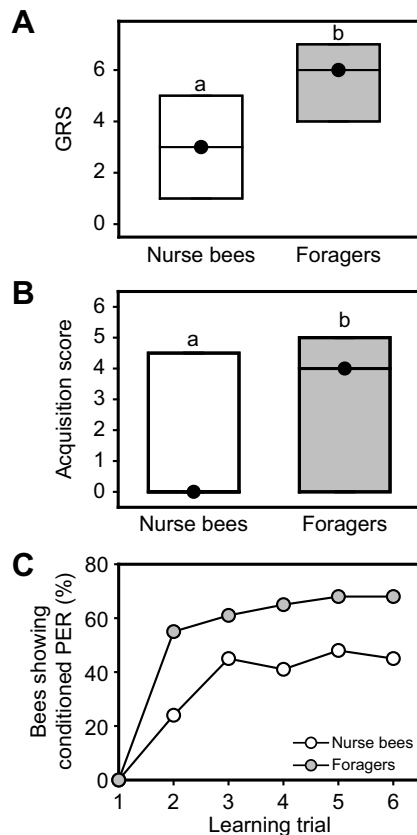


Fig. 2. Gustatory responsiveness and olfactory proboscis extension learning performance of nurse bees and foragers. (A) Median gustatory response scores (GRS, circles) and quartiles (upper and lower lines) of nurse bees and foragers. Foragers display significantly higher GRS than nurse bees ($n_{\text{forager}}=31$, $n_{\text{nurse}}=28$). (B) Median acquisition scores (circles) and quartiles (upper and lower lines) of nurse bees and foragers. Foragers learn better than nurse bees and reach significantly higher acquisition scores ($n_{\text{forager}}=31$, $n_{\text{nurse}}=28$). Different letters indicate a significant difference between groups ($P<0.05$). (C) Acquisition curves of nurse bees and foragers. In both groups, the number of bees showing the conditioned proboscis extension response (PER) increases with learning trials. The learning curves differ almost significantly between nurse bees and foragers, with foragers learning faster and reaching a higher level of learning (logistic regression, $P=0.057$). For details of tests, see Results.

The learning curve of the foragers differed almost significantly from that of nurse bees, with foragers displaying a trend towards faster acquisition and higher learning level (logistic regression, effect of social role: $\chi^2_1=3.64$, $P=0.057$; Fig. 2C).

Tyramine can increase gustatory responsiveness and improve learning in nurse bees

We hypothesized that if the differences in gustatory responsiveness and learning of nurse bees and foragers were related to different tyramine brain titers, treating bees with tyramine should not only elevate their brain titers of tyramine but also increase their gustatory responsiveness and possibly improve their learning performance. Injection of tyramine into the hemolymph significantly elevated tyramine brain titers ($t=3.00$, $P<0.05$, $n_{\text{PBS}}=9$, $n_{\text{tyramine } 10^{-2} \text{ mol l}^{-1}}=7$), but did not affect octopamine brain titers 30 min after injection ($t=0.66$, $P>0.05$, $n_{\text{PBS}}=10$, $n_{\text{TA } 10^{-2} \text{ mol l}^{-1}}=7$; Fig. 3A).

As predicted, injection of tyramine into the hemolymph significantly increased gustatory response scores in nurse-aged honeybees ($P<0.001$; Fig. 3B). Both concentrations of tyramine led to a significant increase in gustatory scores compared with the

control group (TA $10^{-3} \text{ mol l}^{-1}$ versus control: $P<0.01$; TA $10^{-2} \text{ mol l}^{-1}$ versus control: $P<0.001$; $n_{\text{control}}=33$, $n_{\text{TA } 10^{-3} \text{ mol l}^{-1}}=32$, $n_{\text{TA } 10^{-2} \text{ mol l}^{-1}}=32$; Fig. 3B). However, the higher concentration induced a slightly stronger effect and was therefore chosen for further experiments.

Yohimbine is an effective antagonist of honeybee tyramine receptors (Reim et al., 2017) but does not block honeybee octopamine receptors (Balfanz et al., 2014). To test whether the tyramine-induced effect on gustatory responsiveness in nurse bees could be attributed to activation of tyramine receptors, we also tested effects of yohimbine on gustatory responsiveness. In foragers, where tyramine significantly increases sucrose responsiveness (Scheiner et al., 2002), yohimbine can effectively inhibit the tyramine-induced increase in gustatory responsiveness (Fig. S1).

The increase in gustatory responsiveness of nurse bees observed after tyramine treatment disappeared when yohimbine was added to the tyramine solution (TA $10^{-2} \text{ mol l}^{-1}$ versus control: $P<0.001$; TA $10^{-2} \text{ mol l}^{-1}$ +yohimbine $10^{-2} \text{ mol l}^{-1}$ versus control: $P>0.05$, $n_{\text{control}}=33$, $n_{\text{TA } 10^{-2} \text{ mol l}^{-1}}=32$, $n_{\text{TA } 10^{-2} \text{ mol l}^{-1}+\text{yohimbine } 10^{-2} \text{ mol l}^{-1}}=32$; Fig. 3C), suggesting that the behavioral changes induced in nurse bees were due to activation of tyramine receptors.

Because bees with a high gustatory responsiveness generally perform better in associative appetitive learning assays than bees with lower responsiveness (for review, see Scheiner et al., 2004), we hypothesized that nurse bees treated with tyramine should improve their appetitive learning performance. Tyramine-treated nurse-aged bees indeed reached significantly higher learning scores than respective controls ($Z=2.44$, $P<0.05$, $n_{\text{control}}=29$, $n_{\text{TA } 10^{-2} \text{ mol l}^{-1}}=30$; Fig. 3D), and therefore reached a learning performance that was comparable to that of foragers (Fig. 2B). The better learning performance of tyramine-treated nurse bees also becomes apparent in a learning curve, which is significantly different from that of the control (Fig. 3E). Both tyramine-treated nurse bees and controls increasingly displayed the conditioned PER with increasing learning trials (logistic regression, effect of trial: $\chi^2_4=15.77$, $P<0.01$). However, tyramine-treated nurse bees learned faster and reached higher learning rates than the control bees (logistic regression, effect of treatment: $\chi^2_1=5.42$, $P<0.05$).

Finally, we investigated whether we could improve associative learning performance of foragers in the same way as in nurse bees. Injection of tyramine significantly increased gustatory responsiveness in foragers ($Z=2.95$, $P<0.01$, $n_{\text{control}}=31$, $n_{\text{TA } 10^{-2} \text{ mol l}^{-1}}=33$; Fig. 4A), similar to nurse bees. However, the increase in responsiveness was only about half as strong in foragers as in nurse bees. This increase in GRS was apparently not large enough to induce significant improvements of associative learning performance, because foragers treated with tyramine did not differ in their learning scores from control foragers ($Z=0.31$, $P>0.05$, $n_{\text{control}}=57$, $n_{\text{TA } 10^{-2} \text{ mol l}^{-1}}=56$; Fig. 4B). The learning curve of tyramine-treated foragers did not differ from that of control bees (logistic regression, effect of group: $\chi^2_1=0.08$, $P>0.05$). In both groups, the percentage of bees showing the conditioned PER increased with increasing number of learning trials (logistic regression, effect of trial: $\chi^2_4=14.82$, $P<0.01$).

DISCUSSION

Social role, gustatory responsiveness and appetitive learning

Social role correlates with gustatory responsiveness and learning performance. Honeybee foragers performed significantly better in our associative PER learning experiments than nurse bees (Fig. 2B),

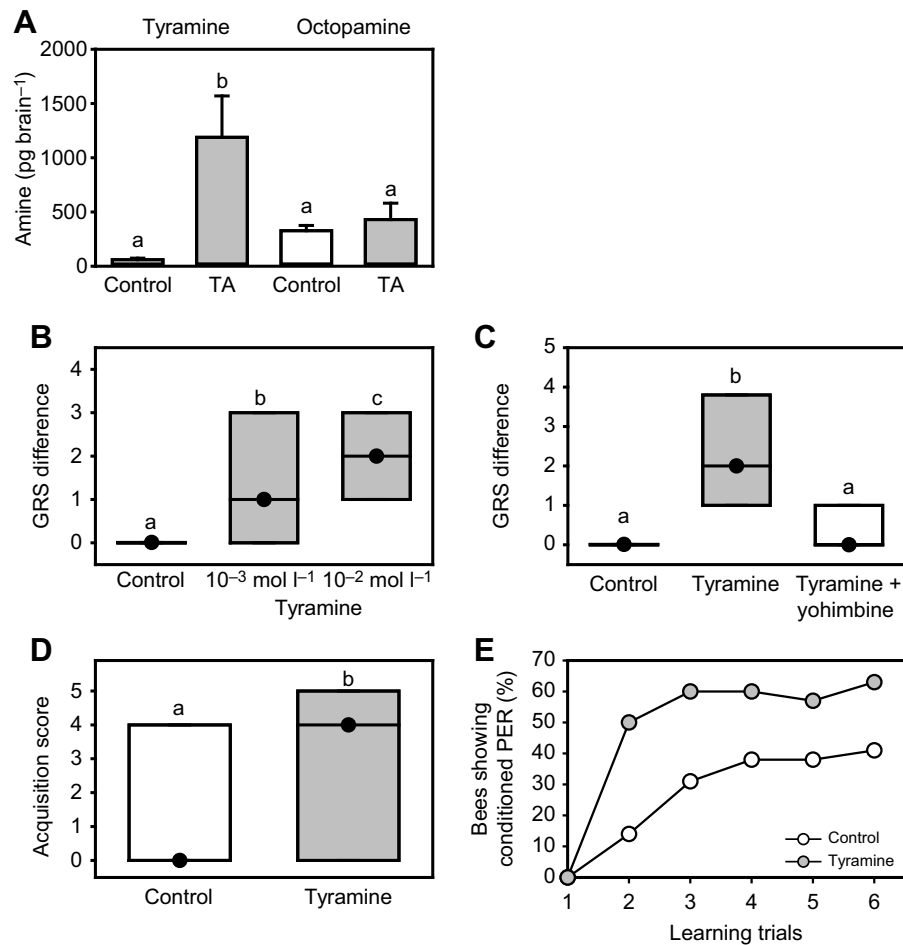


Fig. 3. Modulation of gustatory responsiveness and learning performance in nurse bees by tyramine application. (A) Increase in tyramine brain titers but not in octopamine brain titers 30 min after injection of 1 μ l tyramine (10^{-2} mol l⁻¹) into the hemolymph (tyramine titer: $n_{\text{control}}=9$, $n_{\text{tyramine}}=7$, octopamine titer: $n_{\text{control}}=10$, $n_{\text{tyramine}}=7$). Mean \pm s.e.m. of control and tyramine-injected (TA) bees are displayed. (B) Change in gustatory response scores (GRS) of nurse bees following injection with PBS (control) or different concentrations of tyramine into the hemolymph. ‘GRS difference’ is the difference in GRS measured 30 min after treatment and GRS measured prior to treatment. A positive value indicates that bees become more responsive following treatment. Both concentrations of tyramine significantly increase GRS ($n_{\text{control}}=33$, $n_{\text{TA } 10^{-3} \text{ mol l}^{-1}}=32$, $n_{\text{TA } 10^{-2} \text{ mol l}^{-1}}=32$). (C) Change in GRS of nurse bees following treatment with tyramine (10^{-2} mol l⁻¹) or co-treatment of tyramine (10^{-2} mol l⁻¹) and the tyramine receptor antagonist yohimbine (10^{-2} mol l⁻¹). The tyramine-induced increase in GRS is inhibited by co-injection with yohimbine ($n_{\text{control}}=33$, $n_{\text{TA } 10^{-2} \text{ mol l}^{-1}}=32$, $n_{\text{co-injection}}=31$). (D) Acquisition scores of nurse bees following tyramine (10^{-2} mol l⁻¹) treatment and of controls. Tyramine strongly improved learning performance of nurse bees, which is reflected by significantly higher acquisition scores ($n_{\text{control}}=29$, $n_{\text{TA } 10^{-2} \text{ mol l}^{-1}}=30$). Medians (dots) and quartiles (upper and lower lines) are displayed in B–D. (E) Acquisition curves of nurse bees treated with PBS (control) or tyramine 10^{-2} mol l⁻¹. In both groups, the percentage of bees showing the conditioned PER increases with learning trials. However, the learning curves differ significantly between nurse bees treated with tyramine 10^{-2} mol l⁻¹ and control bees, with tyramine-treated nurse bees learning faster and reaching a higher level of learning. Number of bees tested as in D. Different letters indicate a significant difference between groups ($P < 0.05$). For details of tests, see Results.

most likely because they were also more responsive to gustatory stimuli than nurse bees (Fig. 2A). A number of earlier experiments have suggested a direct link between gustatory responsiveness and associative appetitive learning in honeybees, with bees displaying a higher gustatory responsiveness learning faster and reaching higher acquisition levels than bees with lower responsiveness (Scheiner et al., 1999, 2001a,b, 2005). The idea behind this correlation is that bees with higher gustatory responsiveness subjectively place a higher value on a specific sugar water reward (i.e. the unconditioned stimulus, US) than bees with lower responsiveness (Scheiner et al., 2005). Individual reward evaluation, in turn, could be interpreted as the bee’s intrinsic motivation to learn (Scheiner et al., 1999, 2005). Interestingly, this correlation does not seem to apply to old foragers (i.e. foragers with a long foraging experience, regardless of age). This group is unique in that old foragers have a high gustatory responsiveness but still perform poorly in appetitive PER learning (Behrends et al., 2007; Scheiner and Amdam, 2009; Tolfsen et al.,

2011). Newly emerged honeybees, in contrast, mainly display a poor appetitive learning performance, because they are too unresponsive to sucrose (Behrends and Scheiner, 2009).

Tyramine can improve learning performance by increasing the subjective reward value

A decisive question in neuroscience is how the learning performance of an individual can be improved. Based on our findings above, it should be possible to improve appetitive learning performance by pharmacologically increasing gustatory perception, i.e. perception of the sucrose reward in a water solution. Indeed, we could demonstrate that increasing gustatory responsiveness through pharmacological activation of octopamine receptors suffices to improve appetitive learning performance in young bees (Behrends and Scheiner, 2012). Our current results provide further support for the hypothesis that there is a causal relationship between gustatory responsiveness and appetitive PER learning in honeybees by

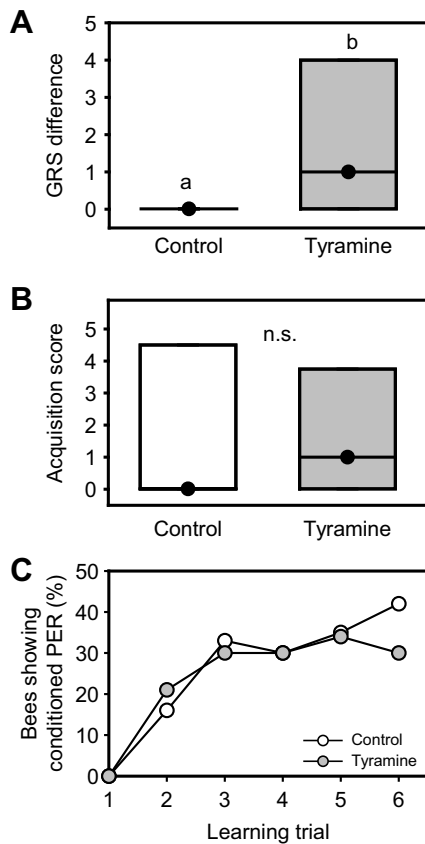


Fig. 4. Modulation of gustatory responsiveness and learning performance in foragers by tyramine application. (A) Change in GRS of foragers following treatment with PBS (control) or tyramine (10^{-2} mol l $^{-1}$). Tyramine induces a significant increase in GRS in foragers ($n_{\text{control}}=31$, $n_{\text{TA } 10^{-2} \text{ mol l}^{-1}}=33$). (B) Acquisition scores of foragers following tyramine (10^{-2} mol l $^{-1}$) treatment and of controls. In contrast to nurse bees, tyramine treatment did not improve learning performance in foragers (n.s.; $n_{\text{control}}=57$, $n_{\text{TA } 10^{-2} \text{ mol l}^{-1}}=56$). Medians (circles) and quartiles (upper and lower lines) are displayed in A and B. Different letters indicate a significant difference between groups ($P<0.05$). (C) Learning curves of foragers treated with tyramine 10^{-2} mol l $^{-1}$ and of controls. In both groups, the percentage of bees showing the conditioned PER increases with learning trials. Foragers treated with tyramine 10^{-2} mol l $^{-1}$ did not differ in their learning curve from controls. For details of tests, see Results.

showing that tyramine treatment not only increases gustatory responsiveness but also improves appetitive learning performance in young bees. Because the tyramine receptor blocker yohimbine inhibits the effects of tyramine on gustatory responsiveness, it should also inhibit the tyramine-induced improvement of learning behavior. However, this hypothesis has yet to be explored experimentally. Although we cannot exclude effects of tyramine on the perception of olfactory stimuli (i.e. the conditioned stimulus, CS) in our assay, effects of individual differences in CS perception on learning performance are generally much smaller than effects of subjective US strength (Scheiner et al., 2005). However, a higher responsiveness to odors may have added to the better learning performance of tyramine-treated bees. In the *Drosophila* mutant *honoka*, in which expression of the tyramine receptor 1 is greatly reduced, olfactory learning performance is strongly impaired (Kutsukake et al., 2000). Therefore, activation of tyramine receptors through elevating tyramine brain levels in our experiments might also increase olfactory perception in honeybees, but we have yet to explore this hypothesis.

Function of tyramine receptors in gustatory responsiveness and appetitive learning

How the pharmacological increase in tyramine titers led to enhanced gustatory responsiveness and improved learning performance is unclear. We assume that tyramine injection, which significantly increased tyramine but not octopamine levels in the brain, activated specific tyramine receptors in the brain of the bees and that increased tyramine signaling enhanced perception of gustatory stimuli. Thus, tyramine would act similarly to octopamine in this context. This is intriguing, as it is generally assumed that octopamine and tyramine act antagonistically (Roeder, 2005). We suggest that the behavioral effects of tyramine strongly depend on the activation of specific tyramine receptors, which may act in a similar way to octopamine receptors but which may also have opposite effects, depending on the signaling cascades involved and on the specific tissues involved in the control of a certain behavior. The behavioral changes of tyramine that are similar to those of octopamine might be achieved through the second honeybee tyramine receptor, AmTYR2, which acts similarly to the octopamine receptors AmOCT β R1 to AmOCT β R4 (Balfanz et al., 2014; Reim et al., 2017) by upregulating cAMP. Octopamine similarly improves appetitive learning performance in honeybees, as does tyramine (Behrends and Scheiner, 2012). However, these are pure speculations. Only targeted knockdown of specific tyramine receptors through RNA interference in the brain (Farooqui et al., 2003) or null mutations of tyramine receptor genes by CRISPR/Cas9 (Kohno et al., 2016) might eventually connect individual receptor types to specific behaviors.

The effects of tyramine depend on social role

Our experiments in nurse bees suggest that tyramine can improve appetitive learning performance largely by increasing responsiveness to the reward. However, foragers did not improve in their learning performance even though they became more responsive. There are several conceivable explanations for this phenomenon. One possibility is that although tyramine increased gustatory responsiveness significantly in foragers, the induced increase was not sufficient to lead to a significant improvement of appetitive learning performance. Secondly, tyramine could act differently in foragers compared with nurse bees, because foragers have a different tyramine receptor gene expression (Fig. 1D) and different tyramine brain titers (Fig. 1C). Tyramine titers in foragers may have reached saturation, so that a further increase in brain titers had no positive impact on learning performance, even though it still increased gustatory responsiveness. Tyramine might further have independent effects on gustatory responsiveness and learning performance, which only become apparent in foragers. Interestingly, octopamine can improve the learning performance of young bees but not of old foragers, very similar to tyramine and even though octopamine increases gustatory responsiveness in old foragers (Behrends and Scheiner, 2012). It is therefore conceivable that after a certain time window in adult development, tyramine and octopamine may not be able to affect cognitive-like functions any longer, even though they can modulate gustatory responsiveness.

Function of tyramine receptors in the nurse–forager transition

Social role and behavioral differences between nurse bees and foragers correlated with variation in tyramine brain titers and mRNA expression of one of the two honeybee tyramine receptors in

the brain. These findings suggest that tyramine signaling changes during behavioral stage transitions in adult honeybees. Biogenic amine titers generally seem to increase in adult development, because foragers also have higher levels of octopamine, serotonin and dopamine than nurse bees (Wagener-Hulme et al., 1999; Schulz and Robinson, 1999). However, in contrast to octopamine, tyramine does not appear to directly release foraging behavior. While pharmacological activation of octopamine receptors induced foraging behavior in nurse bees, tyramine did not have such an effect (Schulz and Robinson, 2001). The exact role of tyramine receptors in the nurse–forager transition is currently unknown. The reduced mRNA expression of *Amyr2* in foragers compared with nurse bees might either be directly related to the nurse–forager transition or be a result of the changed sensory experience of foragers compared with nurse bees. To investigate the role of individual tyramine receptors in the nurse–forager transition of honeybees, a targeted knockdown of the respective receptor is necessary, which has been demonstrated successfully for octopamine receptors (Faroqui et al., 2003). Also, it has to be studied when during the nurse–forager transition the change in *Amyr2* gene expression occurs, i.e. prior to leaving the hive or as a result of the novel experiences by the foragers.

Whether the social-role-related differences in tyramine brain titer or tyramine receptor expression are causally linked to changes in sensory responsiveness during the nurse–forager transition is still unclear. However, tyramine was shown to affect sensory response thresholds for visual and gustatory stimuli (Scheiner et al., 2002, 2014b) and also affected locomotion (Scheiner et al., 2014b). As foragers and nurse bees differ naturally in all of these behavioral responses, we suggest that tyramine plays a role in modulating sensory responsiveness and locomotor activity to a larger extent than assumed hitherto. One alternative explanation is that the increased brain titer of tyramine found in foragers is related to the fact that foragers also have higher octopamine titers than nurse bees (Schulz and Robinson, 1999). As tyramine is the metabolic precursor of octopamine, the higher tyramine titer might not directly cause behavioral changes, but additional tyramine may be converted into octopamine. Similar to tyramine, octopamine was shown to increase gustatory responsiveness in honeybees (Scheiner et al., 2002; Behrends and Scheiner, 2012). Ultimately, experiments manipulating the expression of specific tyramine and octopamine receptors (Faroqui et al., 2003) will help to separate the role of octopamine and tyramine and to attribute behavioral changes during the nurse–forager transition to specific receptor types.

In summary, our data suggest an important role for the neurotransmitter tyramine in regulating and modulating sensory responsiveness, appetitive learning and age-dependent division of labor in honeybees and other insects.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

R.S., A.B.B., M.T. and T.R. designed the study. R.S. was in charge of the behavioral experiments, T.R., E.S. and B.V.E. performed the HPLC experiments. M.T. performed the qPCR experiments. All authors were involved in analyzing the data. R.S. wrote the first draft of the manuscript. All authors contributed to the final version of the manuscript.

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Supplementary information

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