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ORIGINAL PAPER

The role of tyramine and octopamine in the regulation of reproduction in queenless worker honeybees

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Abstract In honeybees, workers under queenless condition compete for reproduction and establish reproductive dominance hierarchy. Ovary activation is generally accompanied by the expression of queen-like pheromones. Biogenic amines (BAs), in particular dopamine, are believed to be involved in this process by regulating ovarian development. However, the role of BAs in establishing reproductive dominance or their effect on queen-like pheromone production was not investigated. Here, we explored the effect of octopamine (OA) and tyramine (TA) oral treatments on the propensity of treated bees to become reproductively dominant and produce queen-like pheromones in Dufour's and mandibular glands. One bee in a pair was treated with either OA or TA while the other was fed sugar solution. TA was found to enhance ovary development and the production of esters in the Dufour's gland and 9HDA (queen component) in the mandibular glands, thus facilitating worker reproductive dominance. OA, on the other hand, did not enhance ovarian development or ester production, but increased the production of 10HDA (worker major component) in the mandibular glands of their sugar-paired mates. OA is known to induce foraging behavior by workers, while increased production of 10HDA characterizes nursing workers. Therefore, we suggest that TA induces reproductive division of labor, while OA treatment results in caste differentiation of workers to foragers and nurses.

Keywords Reproductive division of labor · Biogenic amines · Honeybees · Workers · Pheromones · Dominance

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Introduction

Reproductive division of labor is a hallmark in the evolution and organization of insect societies. In honeybees (*Apis mellifera*), reproduction is an attribute of the queen, whereas workers perform tasks related to the organization and maintenance of the hive. This reproductive skew clearly depends on the presence of the queen and the expression of her pheromones. In a queenless colony, some but not all of the workers become laying workers and can inhibit ovary development in other workers (Crewe and Velthuis 1980; Page and Erickson 1988). The major pheromone-producing glands are the mandibular and Dufour's glands. The mandibular glands are important in suppression of worker reproduction (Hoover et al. 2003; Winston et al. 1990) and are involved in the establishment of reproductive hierarchy among queenless (QL) workers (Malka et al. 2008; Moritz et al. 2000, 2004). Dofour's gland secretion is highly correlated with ovarian development and thus serves as a fertility signal (Dor et al. 2005; Katzav-Gozansky et al. 2004). While the pheromonal bouquet is identified with queens, workers express queen-like pheromones in association with ovarian development (Dor et al. 2005; Katzav-Gozansky et

al. 2004 for Dufour's gland; Crewe and Velthuis 1980; Plettner et al. 1993 for mandibular glands). Malka et al. (2008) suggested that the mandibular and Dufour's glands are important in establishing reproductive dominance among QL workers. When the colony becomes QL, workers compete over reproduction. Some workers start producing queen-like pheromones in the mandibular glands, inhibiting ovarian development in other workers (Sakagami 1954), while others produce a disproportionate amount of pheromones in their Dufour's gland as fertility signal and initiate ovary development (Malka et al. 2008).

The transition of workers in QL colonies from normal to reproductive workers result from physiological changes in the brain that leads to initiation of oviposition (Page and Erickson 1988) and dominance (Moritz and Hillesheim 1985). These changes may be mediated by brain biogenic amines (BAs), important neuromodulators, neurohormones, and neurotransmitters (reviewed in Blenau and Baumann 2001). BAs affect multiple physiological and behavioral processes in honeybees (Orchard 1982; Roeder 1994, 1999, 2005; Scheiner et al. 2002), in particular with respect to behavioral maturation of workers, their reproductive physiology, and behavior. Octopamine (OA) seems to be the major BA mediator of worker behavioral maturation. Its brain levels in foragers are higher than in nurses (Wagener-Hulme et al. 1999), and treating 4-day-old bees with OA resulted in precocious foraging (Schulz and Robinson 2001; Schulz et al. 2002). Nonetheless, Taylor et al. (1992) showed that foragers had significantly elevated levels of dopamine (DA) in their brains compared to younger bees performing in hive tasks such as nursing or food storing. With respect to reproductive division of labor, queens were shown to have higher levels of DA in their brain compared to workers, irrespective of the size of the brain (Brandes et al. 1990; Harano et al. 2005), suggesting a role of DA in reproduction. Additionally, QL workers with developed ovaries possess higher levels of DA and tyramine (TA) than QR workers showing undeveloped ovaries, the latter presumable through the augmentation of DA (Sasaki and Nagao 2001 for DA; Sasaki and Harano 2007 for TA). Ovary development can be enhanced in QL workers by treatment with DA (Dombroski et al. 2003), showing the strong correlation between DA and ovary development. Brain measurements of BAs in sterile vs. fertile as well as dominant and subordinate workers in the bumblebee *Bombus terrestris* indicate that DA is associated with ovarian development whereas OA is involved in the establishment of dominance hierarchy (Bloch et al. 2000). So far, studies showed the importance of either BAs or pheromone production in reproductive division of labor. The queen mandibular pheromone (QMP), in particular, its homovanillyl alcohol component, seems to be responsible for maintaining low DA levels in the workers' brain, thus presumably leading to sterility (Beggs et al. 2007). It was further postulated that QMP, via its effect on the expression of some DA receptors, influences the attraction of workers to the queen (Vergoz et al. 2009). Barron et al. (2002) showed that OA increases the responsiveness of honeybees to brood pheromone (an activator of foraging), by increasing the activity level of foragers. Thus, the authors suggest that OA acts as a neuromodulator, changing the response threshold to task stimuli among bees. However, studies pertaining to the possible role of brain BAs in pheromone production are needed. The present study aims at exploring the role of two BAs, octopamine and tyramine, in reproductive dominance establishment among queenless workers. To that effect, we treated bees with orally applied BAs and examined the levels of the mandibular and Dufour's gland pheromones relative to their ovarian development. We hypothesized that these BAs may increase the propensity of the treated workers to become the reproductively dominant bee.

Material and methods

Bees and experimental setup

Callow workers (less than 24 h old) were obtained from six commercial hives kept in the I. Meier Segals Garden for Zoological Research at Tel Aviv University, Israel, during 2009. They were collected from sealed brood combs placed in a temperature-controlled room for 24 h (33°C and 60% humidity) and divided into treatments so that in each treatment, only approximately four pairs originated from the same hive. For testing the effect of the BAs tyramine and octopamine on reproductive dominance establishment, we kept pairs of callow bees in a petri dish (9 cm in diameter) lined with filter paper in the temperature-controlled room for 14 days, allowing the establishment of reproductive dominance (Dor et al. 2005). Each bee in a pair was color-marked according to the BA treatment. BA administration was achieved through feeding, which proved effective in honeybees (Dombroski et al. 2003). Each bee daily received either 2 μg of the BA (either octopamine or tyramine) in 50 μl of 60% sugar solution or pure sugar syrup (see controls below) (Barron et al. 2007). Before each feeding period, the bees were starved for 2 h to increase their feeding probability. During feeding, the two bees were separated by a divider, allowing us to provide one bee with the BA solution and the other with sugar solution according to their marking. The bees were allowed to feed on the solution for 2 h, after which the amount of solution left was measured using a 10-μl Hamilton syringe, and distilled water was provided ad lib. The bees were kept isolated for an additional 2 h, to minimize the probability of BA transfer by trophallaxis. Afterwards, the divider was removed and both bees were

allowed to interact freely. Both were provided with a pollen cake ad lib. We performed two controls: The first control consisted of pairs that were manipulated as above except that both received pure sugar during the separation period (henceforth sugar-fed control (SU)). The second control constituted of bees that received 60% sugar solution ad lib and left undisturbed without feeding time limitation or a divider (henceforth undisturbed control). At the end of the experiment, bees were immediately placed in −80°C freezer until dissection and analysis. For comparing the reproductive performance of the sugar-fed and undisturbed controls, the bees in each pair were classified as dominant or subordinate according to their level of ovary development. For comparing the BA treatments and sugar-fed control, the latter were classified as dominant or subordinate at random (for further explanation see "Results"). Results of the level of ovarian development and pheromone levels are presented as the delta between the BA-treated and sugar-fed bees of each pair. Outliers were removed when necessary.

Dissections and chemical analyses

For brain BA quantification and mandibular gland pheromone analyses, frozen heads were dissected under a dissecting microscope on dry ice. The brain was carefully removed and placed in 100 μl of methanol. Quantification of brain BAs was done as described in Barron et al. (2007).

The mandibular glands were excised and immediately extracted in methanol containing decanoic acid (0.1 μg/μl) as the internal standard. The samples were then evaporated to dryness and silylated with 25 μl BSTFA (*N,O*-bis(trimethylsilyl)trifluoroacetamide) + TMS (trimethylchlorosilane) (Supelco, 3-3148) after which their constituents were quantified by gas chromatography (GC), using peak integration in comparison to the internal standard.

For assessing ovarian development and analysis of Dufour's gland secretion, the bees' abdomens were dissected under a dissecting microscope in distilled water. The ovaries of each bee were removed, and the terminal oocyte in each ovariole (being the most mature oocyte) was measured using a calibrated ocular. The Dufour's gland was carefully separated from the sting apparatus, extracted in 50 μl dichloromethane containing eicosane (100 ng/50 μl) as an internal standard and stored at −20°C. Quantitative analyses were conducted by GC (Varian CP 3800) using a VM-5 fused silica column that was temperature-programmed from 150°C (1 min of initial hold) at 5°C/min to 300°C with a final hold of 10 min (Katzav-Gozansky et al. 1997). Compound quantification was done by peak integration in comparison to the internal standard. Chemical identification of both the mandibular and Dufour's glands' constituents was verified using GC/MS and in comparison to standards of each of the major components.

Statistical analysis

Variables measuring differences between pairs were analyzed using paired *t* test or Wilcoxon signed rank test when the data did not distribute normally. Similarly, analysis of variance with Tukey comparisons or Kruskal–Wallis analyses with Steel–Dwass comparisons were used to compare the difference (Δ) between BA and sugar-fed bees among treatments. Comparison of the difference to zero was conducted by one-sample *t* test. Variables measuring differences between the control treatments were analyzed using Student's*t* test or Mann–Whitney *U* test when the data did not distribute normally. All variables were checked for normality followed by either logarithmic or square root transformations when necessary. All statistical tests were two-tailed, with significance level of 0.05. Analyses were conducted using Systat 11 (SPSS, Inc., Chicago, IL, USA), except the Steel–Dwass test which was conducted with JMP 9 (SAS, Cary, NC, USA).

Results

Our BA treatment did not result in a significant difference in brain BA levels between paired bees (Table 1), although bees receiving TA showed higher values of brain TA or OA than their sugar-paired bee.

To determine whether paired bees can establish reproductive dominance, we calculated the difference in maximum oocyte size between the dominant (the bee with higher oocyte size) and subordinate bee for each pair in both the undisturbed and sugar-fed controls; a significant positive value indicates dominance establishment. The difference in ovarian development was significantly different from zero for both control treatments (Fig. 1a: one-sample *t* test: undisturbed: $n=23$, $t=4.7$, $p<0.0001$; sugar-fed control: $n=24$, $t=4.3$, $p<0.0001$), although undisturbed bees achieved a higher reproductive dominance than sugar-fed bees, indicated by the greater differences in ovarian development (Mann–Whitney: $n=47$, $u=367$, $p=0.050$). This shows that the separation of the sugar-treated bees by the divider (i.e., sugar control) did not affect their ability to establish dominance, which is also supported by a significant difference in their oocyte size within the pair (0.2± 0.04 and 0.08±0.02 mm; Wilcoxon signed rank test: $n=48$, $z=-3.5$, $p<0.0001$), as was the case without a divider (i.e., undisturbed control) (0.62±0.12 and 0.29±0.09 mm; $n=46$, $z=-3.72$, $p<0.0001$). This is strengthened by the significant difference in ovarian development between the dominant bees in each of the control treatments ($n=47$, $u=391.5$, $p=0.013$).

Pair-wise comparisons of oocyte size revealed larger oocyte size for TA-treated bee relative to its pair mate, but no significant differences between OA- and SU-fed bees and their pair mates (Table 1). To assess the effect of the BA

Table 1 Summary of results of brain biogenic amines (BA), ovary development, and pheromones produced by paired bees receiving either octopamine (OA), tyramine (TA), or sugar (SU) compared to their paired mate receiving only sugar

	OA pairs					TA pairs					SU control pairs				
Variable	Drug	Sugar	Number	*t/z*	*p*	Drug	Sugar	Number	*t/z*	*p*	Sugar 1	Sugar 2	Number	*t/z*	*p*
Brain OA (ng)	0.8±0.1	1.1±0.2	13	−1.1	0.28	1.2±0.25	0.7±0.1	13	1.4	0.17	0.9±0.1	1.07±0.2	14	0.78	0.45
Brain TA (pg)	12.8±3.8	9.3±2.6	13	1.03	0.32	18.3±5.4	11.2±0.2	13	1.3	0.19	16.9±5.3	12.5±2.6	14	−0.29	0.77
Oocyte size (mm)	0.2±0.06	0.2±0.07	22	−0.04	0.96	0.3±0.1	0.11±0.03	28	−2.02	**0.044**	0.2±0.06	0.1±0.04	26	−0.46	0.64
Esters (ng)	69.3±41.4	115.6±57.3	10	−0.68	0.51	47.9±14.3	10.6±3.7	20	−2.5	**0.023**	81.1±31.5	125.9±52	15	−0.51	0.61
9HDA (μg)	3.7±0.7	3.1±0.8	10	−1.4	0.18	3.9±0.8	3.04±0.8	15	2.2	**0.047**	3.9±0.8	2.7±0.6	19	1.18	0.25
10HDA (μg)	27.4±5.3	49.5±8.1	10	−2.18	0.051	46.5±6.2	30.6±4.8	15	1.9	0.074	36.8±4.5	26±5.7	17	1.6	0.13

SU control pairs are sorted at random. All comparisons were done by paired *t* test, except for "Oocyte size" which did not distribute normally and therefore analyzed by Wilcoxon signed rank test
Significant values are in bold

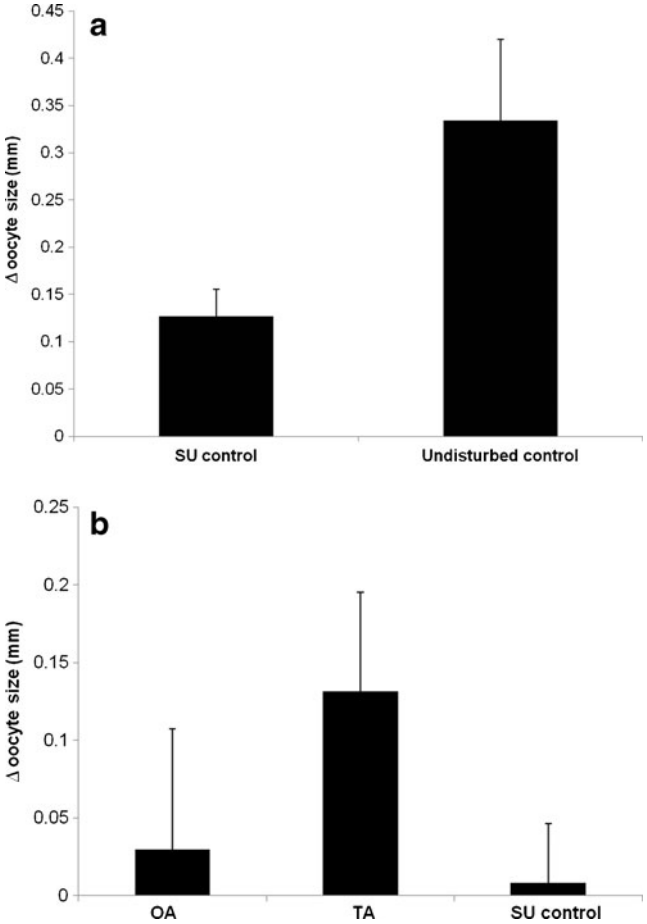

Fig. 1 The difference in oocyte size between pairs in the (**a**) sugar control and (**b**) BA treatments of octopamine (*OA*), tyramine (*TA*), or sugar (*SU*). **a** Undisturbed bees (i.e., without a divider) and sugar control (with a divider) bees are sorted according to ovarian development. The difference in oocyte size was calculated by subtracting the value of the bee with undeveloped ovaries from that with developed ovaries. Both control treatments show significant difference from zero ($p<0.0001$ for both), with undisturbed pairs showing higher difference in ovarian development than sugar control pairs ($p=0.050$). **b** BA treatments are sorted according to BA and sugar-paired mate while the SU control pairs are sorted at random. The difference in oocyte size was calculated by subtracting the value of the sugar-paired mate from the BA-treated bee. Although there was no significant difference among treatments ($p=0.45$), the difference in oocyte size differed significantly from zero between the two paired bees only for TA ($p<0.0001$ for both), but not for OA ($p=0.7$) and SU control pairs ($p=0.8$). *Error bars* show standard error

treatments on the propensity of the treated bee to become dominant, we compared the differences in oocyte size between the treated bee and its sugar-fed pair mate as above. There was no significant difference among the treatments in the difference in oocyte size (Fig. 1b: Kruskal–Wallis: $n=73$, $H=2$, $p=0.37$). Finally, we examined the propensity of bees in each pair to become reproductively dominant, by comparing the differences in oocyte size within a pair to the expected value of zero, since the probability of each of the bees in a pair to become dominant is equal. Values

significantly different from zero means that the BA affects the bee's probability of becoming dominant; positive if the BA facilitates dominance establishment or negative if the BA impedes it. Comparing each of the treatments to the expected zero value (i.e., no effect of BA treatment) revealed that the positive difference between the two paired bees differed significantly from zero for TA-treated bees (mean±SE, 0.13±0.06; n=27, t=16.5, p<0.0001), but not for OA (0.03±0.07; n=22, t=0.38, p=0.7) and sugar-paired bees (0.008±0.04; n=24, t=0.21, p=0.83). This shows that bees treated with TA had a higher probability of activating ovaries than their sugar-paired mate.

Undisturbed pairs showed a larger difference in ester production than sugar control bees (Fig. 2a: n=30, u=167, p=0.022). Undisturbed bees with developed ovaries produced more esters (119.07±29.3 ng) than their paired mates with less developed ovaries (39.7±15.57 ng; n=15, t=−2.8, p=0.014). Sugar control pairs showed no significant difference in ester production between ovary developed and less developed paired bees (46.46±15.6 and 41.8±17.06 ng, respectively; n=15, t=0.3, p=0.76).

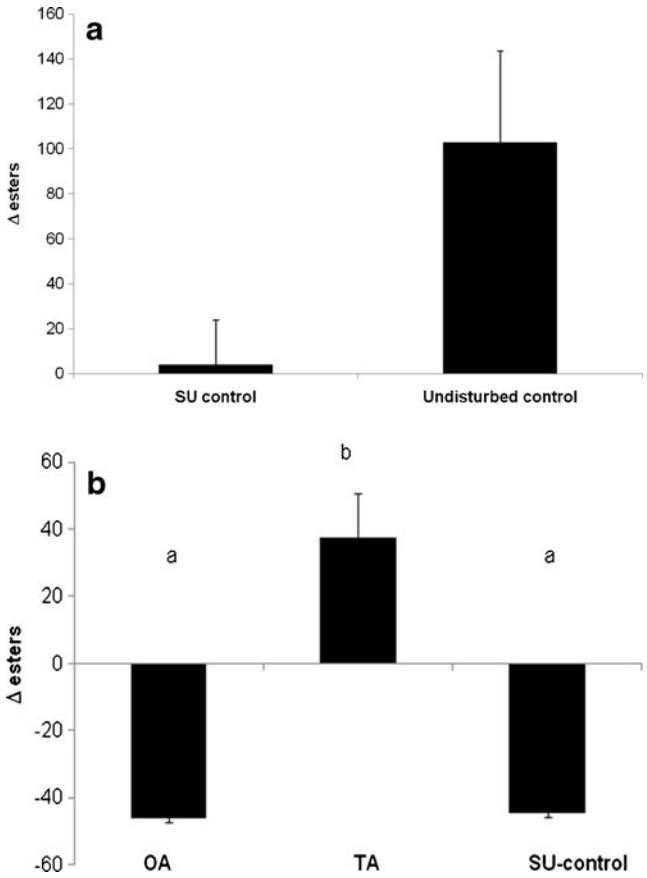

Fig. 2 The difference in ester production in the Dufour's gland of **a** sugar control pairs and **b** BA-treated bees receiving octopamine (*OA*), tyramine (*TA*), or sugar (*SU*). The difference was calculated as specified in Fig. 1. Sugar-paired bees are sorted according to ovarian development in **a**, but sorted at random in **b**. **a** Undisturbed bees showed higher ester production than sugar control bees (p=0.022). **b** BA treatment significantly affected ester production (p=0.024) with TA-treated bees showing higher ester production than OA (p=0.050) or SU-treated bees (p=0.046). *Different letters* indicate significant difference between treatments. *Error bars* show standard error

Treatment had a significant effect on ester production (Fig. 2b: Kruskal–Wallis with Steel–Dwass comparisons: n=46, H=8.03, p=0.018) with TA-treated bees showing significantly higher ester production than for OA (n=30, z=2.26, p=0.060) or SU-treated bees (n=36, z=2.37, p=0.046). Accordingly, values for the TA treatment were greater than zero, i.e., higher propoensity of the treated bees to produce esters (n=20, t=2.78, p=0.012). Treatments with OA and SU showed a negative differential production of esters that did not differ significantly from zero (OA: n=10, t=−1.6, p=0.14; SU: n=15, t=−1.14, p=0.27). Pair-wise comparisons showed a siginificant difference in ester production between TA pairs but not OA and SU control pairs (Table 1).

Reproductively dominant bees in the undisturbed control treatment had a lower total amount of mandibular secretion than their paired mate, but this difference was not significant (35.12±5.13 and 51.54±7.4 μg, respectively; n=15, t=−2.05, p=0.06). Sugar control bees showed no difference in total amount of mandibular secretions between the paired bees (49.5±7.01 and 45.5±5.8; n=18, t=0.45, p=0.65). For the BA treatments, we found that the OA-treated bee showed a lower amount of secretions in the mandibular glands than their sugar-paired mate (42.41±5.18 and 69.5±8.4 μg; n=14, t=−2.63, p=0.02). There was no significant difference in the amount of secretions between paired mates neither in the TA treatment (61.8±5.9 and 50.27±5.5; n=21, t=1.39, p=0.18) nor SU control (51.7±6 and 43.3±6.7; n=18, t=0.97, p=0.34).

In order to evaluate better a possible BA effect on the mandibular secretory components, we further examined the amounts of 9HDA (a queen component) and 10HDA (a worker component). Our results indicate that the difference in 9HDA between the two control treatments did not differ significantly (Fig. 3a: n=28, t=−1.17, p=0.25). There was no difference in the amount of 9HDA between pair mates in the undisturbed control (3.02±0.57 and 2.16±0.59, respectively; n=12, t=1.3, p=0.21), and accordingly, the differential amount between paired mates was not different from zero (n=12, t=1.3, p=0.218). On the other hand, dominant bees in the sugar control pairs showed higher amounts of 9HDA (4.31±0.84 μg) than the subordinate bee (2.97±0.82; n=17, t=2.6, p=0.019), and the differential was significantly different from zero (n=16, t=4.22, p=0.001).

Globally, there were no significant differences in Δ9HDA among treatments (Fig. 3b: one-way ANOVA: n=44, $F_{2,\ 41}$=0.114, p=0.89). Neither did the differential production of 9HDA differ from zero in each of the treatments (OA: n=10, t=1.6, p=0.14; TA: n=15, t=1.76, p=0.09; SU:

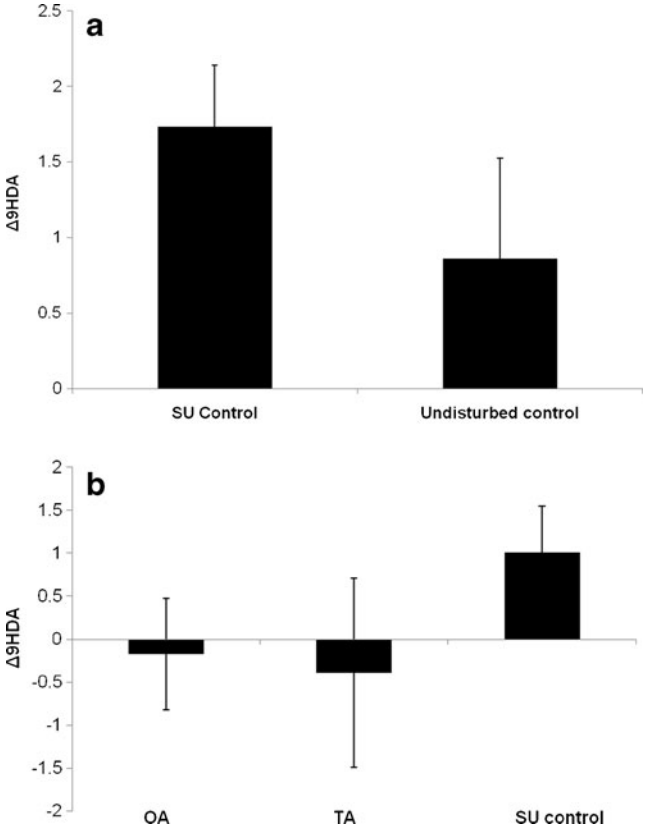

Fig. 3 The difference in the amount of 9HDA produced in the mandibular glands of **a** sugar control pairs and **b** BA-treated bees receiving octopamine (*OA*), tyramine (*TA*), or sugar (*SU*). The difference was calculated as specified in Fig. 1. Sugar-paired bees are sorted according to ovarian development in **a**, but sorted at random in **b**. **a** There was no difference in the amount of 9HDA between the two control treatments (p=0.25) and **b** among the BA treatments (p=0.89). *Error bars* show standard error

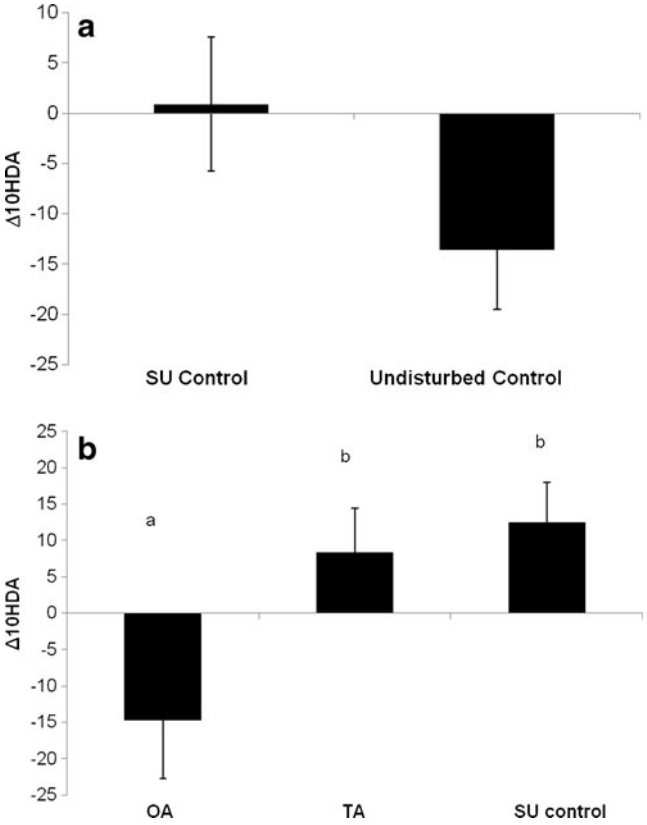

Fig. 4 The difference in the amount of 10HDA produced in the mandibular glands of **a** sugar control pairs and **b** BA-treated bees receiving octopamine (*OA*), tyramine (*TA*), or sugar (*SU*). The difference was calculated as specified in Fig. 1. Sugar-paired bees are sorted according to ovarian development in **a**, but sorted at random in **b**. **a** There was no significant difference in the amount of 10HDA secreted between the control treatments (p=0.19), nor did they differ from zero (p>0.1 for both). **b** BA treatment significantly affected 10HDA production (p=0.009), with OA pairs showing lower difference in 10HDA production than TA pairs (p=0.009) or SU control pairs (p=0.025). *Different letters* indicate significant difference between treatments. *Error bars* show standard error

n=19, t=1.8, p=0.07). Nevertheless, we found a significant difference in the amount of 9HDA secreted between BA and sugar-treated pair mate for the TA treatment, but not for OA treatment nor the SU control pairs (Table 1).

Δ10HDA did not differ between the control treatments (Fig. 4a: n=36, t=1.54, p=0.132), nor did the difference within sugar control pairs differ from zero (sugar control: n=17, t=0.42, p=0.68) or within the pair (30.07±4.6 and 27.06±4.6; n=17, t=0.45, p=0.65). Nevertheless, the difference in 10HDA within undisturbed pairs significantly differed from zero (n=13, t=−1.53, p=0.15), where dominant bees showed lower amounts of 10HDA than their subordinate pair mates (25.68±3.5 and 39.24±5.41, respectively; n=15, t=−2.29, p=0.038).

We found a signficant effect of BA treatments on the differential production of 10HDA between pair mates (Fig. 4b: one-way ANOVA with Tukey comparisons: n=42, $F_{2,\ 39}$=5.38, p=0.009). OA-treated bees showed a lower difference compared to the TA treatment (p=0.009) or SU control pairs (p=0.025). We found a negative, but not significant difference from zero, in 10HDA secretions between OA-treated bees and their paired bees (n=10, t=−2.18, p=0.051), indicating that sugar-fed bees secreted more 10HDA than their BA-treated mates. There was no difference from zero for TA (n=15, t=1.9, p=0.074) and SU pair mates (n=17, t=1.6, p=0.13), which is supported by the lack of difference within these pairs in the amount of 10HDA secreted (Table 1).

Discussion

In this study, we hypothesized that biogenic amines, in particular tyramine and/or octopamine, are mediators of reproductive dominance among worker honeybees. Accordingly, we tested whether we can increase the probability of bees treated with these BAs to become dominant when housed in pairs (Dor et al. 2005). Oral administration of

either OA or TA (Barron et al. 2007) resulted in more brain TA in treated bees compared to their paired mate for both treatments with TA and OA, but these differences were not statistically significant. However, since the levels of brain BAs in our bees were similar to that reported for treated bees in the literature (e.g., Schulz and Robinson 2001), we attribute the lack of significant difference within each pair to higher than expected BA level in the sugar-fed paired mate. Although we made all efforts to prevent any BA transfer through social contact between pair mates (see "Material and methods" and below), there may have been some transfer via trophallaxis between the bees, reducing the differences in BA levels between the treated and sugar-fed bees. If occurred, such transfer did not mask the physiological effect of the BA since the results indicate that the BA administration influenced reproductive division of labor (below). Nevertheless, the similarity in brain BA to treated bees in the literature (e.g., Schulz and Robinson 2001) indicates that the oral treatment was adequate and is likely that BAs reached the hemolymph. Barron et al. (2007) showed that within 15–60 min post delivery, half of the OA was lost from the brain and recovered in the hemolymph. Thus, our BA-treated bees may have had high levels of OA and TA in their hemolymph, acting directly on the peripheral organs including the ovary, Doufor's, and mandibular glands, regardless of their level in the brain. This is supported by the effect of BAs on ovary development and pheromone production in our bees.

Selective BA administration to bees is complicated since they tend to be rapidly metabolized which, therefore, requires chronic administration. TA is the substrate for the synthesis of OA; therefore, administration of TA could lead to increases in OA. Although it is possible that some of the effects observed for TA were due to an increase in OA, it is well established that TA is also a neuroactive substance on its own right (Alkema et al. 2005; Kutsukake et al. 2000; Nagaya et al. 2002). The fact that TA but not OA produced distinct effects on ovary development and pheromonal secretion strengthens the evidence that TA has an independent effect from that of OA. Furthermore, there are specific receptors for OA (AmOA1) and TA (AmTYR1) in honeybees and the development of these receptors occurs by binding of the specific BAs (reviewed in Blenau and Baumann 2001). Therefore, cross-reactivity of the BA receptors is not likely. A further complication was to achieve differential BA feeding of the bees while keeping as much as possible their social environment. To overcome these two obstacles, we had to physically separate the bees for 6 h daily, which may have repercussions on their ovarian development. Comparison of the degree of ovarian development of manipulated and unrestrained bees showed that although in the absence of the divider, bees produced larger terminal oocytes and thus achieved a larger difference in ovarian development within the pair, the manipulated bees also developed reproductive dominance within a pair. However, the lower values achieved in the manipulated bees indicate that constant interactions between the bees are required for effective communication in order to establish a reproductive hierarchy. This conclusion is supported by Dor et al. (2005) that showed that socially restricted bees had less developed ovaries than unrestricted bees, suggesting that tactile and odor communication is important to establish reproductive dominance. In line with previous studies, the reproductively dominant bees produced more esters in their Dufour's gland and more 9HDA (both are queen components) than their paired mates, while the subordinate bees produced more 10HDA (worker component) (Dor et al. 2005; Katzav-Gozansky et al. 2004 for Dufour's gland; Plettner et al. 1993 for mandibular glands).

TA increased the propensity of the treated bee to become reproductively dominant over its sugar-fed pair mate. The null hypothesis was that both bees in a pair have equal probability to become dominant, and therefore, the differences in oocyte size between bees in a pair should not be different from zero. This indeed was the case in the sugar control, where we assigned dominance status to the bees at random, supporting the null hypothesis. In the case of BA treatments, we assigned dominance status to the treated bees, under the null hypothesis that the BA increases the probability of the bees to become dominant. Therefore, a value that is greater than zero corroborates the null hypothesis. In both cases, the values were significantly different from zero, indicating that TA promoted reproductive dominance establishment. These findings are consistent with previous studies in which oral treatments of TA to QL workers accelerated their ovarian development, presumably through stimulating the biosynthesis of DA in the brain that in turn boost ovarian development (Sasaki and Nagao 2002; Sasaki and Harano 2007).

Reproductive honeybee workers also show elevated amounts of queen-specific pheromones both in the mandibular and Dufour's gland (reviewed in Le Conte and Hefetz 2008). Pairs treated with TA showed differential ester production in the Dufour's gland, indicating that TA augments ester production. Since these bees had also greater ovarian development compared to their sugar-fed nestmates and since ester occurrence is correlated with ovarian development (Dor et al. 2005), it is not clear whether TA directly affects ester production or the latter is increased via its effect on ovarian development. However, the fact that the pheromonal and the reproductive system are uncoupled, namely that inhibition of ovarian development does not affect ester levels in Dufour's gland (Malka et al. 2009) lend credence to the hypothesis that TA may affect ester production directly. This hypothesis is further supported by the higher production of 9HDA (a queen mandibular compound), but not 10HDA (a worker mandibular compound) by TA-treated bees rather than their sugar-fed paired mate. Thus, we suggest that TA enhances

ovary development, ester, and 9HDA production, facilitating worker reproduction.

Surprisingly, OA did not have a clear influence on the bees' propensity to become reproductively dominant. This lack of effect strengthens the independent effect of TA on reproductive dominance. High levels of OA were previously shown to occur in the brain of foraging honeybees (Wagener-Hulme et al. 1999), and treatment of nurses with OA induced foraging behavior (Schulz and Robinson 2001; Schulz et al. 2002). In our experiment, sugar-treated pair mates showed higher levels of mandibular secretions than that of OA-treated bees, explained by increased levels of 10HDA. 10HDA is the main fatty acid in the royal jelly fed to the larva (Barker et al. 1959; Weaver et al. 1968) and is considered an important larval nutrient that prevents precocious larval pupation (Kinoshita and Shuel 1975). Therefore, the increased production of 10HDA by sugar-treated pair mates indicates characteristic of nursing workers. Taken together, we suggest that treating pairs with OA encourages caste differentiation between workers, with OA-treated bees acting as foragers while their paired mates are behaving as nurses. As our experimental settings differ from that of a colony, the effect of OA on caste differentiation in natural settings remains to be examined.

Overall, we propose that TA induces reproductive dominance, by regulating the fertility level of the bee, encouraging ovary development, and increasing the production of esters and 9HDA (queen-like pheromone). OA, on the other hand, enhanced the production of worker pheromone (10HDA) in the sugar pair mates, suggesting that OA treatment encourages non-treated bees to act as nurses producing royal jelly for the brood. Since OA is known to induce foraging behavior in workers (Schulz and Robinson 2001; Schulz et al. 2002), we suggest that OA treatment encourages caste differentiation between workers. In light of our results, we suggest that while OA regulates caste differentiation, TA encourages reproductive division of labor by regulating the fertility signals (i.e., ovary development and ester and 9HAD production). Furthermore, we hypothesize that TA and OA work together; increased TA levels lead to reproductive differentiation to egg layers and workers, while OA affects the differentiation to castes within the non-laying workers. More studies on the effect of OA are required to support this hypothesis. Further examination of BAs under natural conditions inside the hive is necessary to reveal whether there are other factors (e.g., brood presence) that may affect the regulation of BAs on reproductive dominance and caste differentiation.

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Ethical note The experiments comply with the current laws of Israel.

Conflict of interest The authors declare that they have no conflict of interest.

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