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RESEARCH ARTICLE

Octopamine and tyramine modulate the thermoregulatory fanning response in honey bees (*Apis mellifera*)

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ABSTRACT

Biogenic amines regulate the proximate mechanisms underlying most behavior, including those that contribute to the overall success of complex societies. For honey bees, one crucial set of behaviors contributing to the welfare of a colony is involved with nest thermoregulation. Worker honeybees cool the colony by performing a fanning behavior, the expression of which is largely influenced by response thresholds modulated by the social environment. Here, we examined how changes in biogenic amines affect this group-performed thermoregulatory fanning behavior in honeybees. Concentrations of two biogenic amines, octopamine and tyramine, are significantly lower in active fanners than in non-fanners, but there is no difference in dopamine and serotonin concentrations. Direct feeding of octopamine and tyramine induced a decrease in fanning responses, but only when both amines were included in the treatment. This is the first evidence that fanning behavior is influenced by these two biogenic amines, and this result is consistent with the typical role of these neurotransmitters in regulating locomotor activity in other insects. Individual variation in amine expression also provides a mechanistic link that helps to explain how this group behavior might be coordinated within a colony.

KEY WORDS: Biogenic amines, Octopamine, Tyramine, Honey bees, Social behavior

INTRODUCTION

Biogenic amines play a significant role in the proximate mechanisms of behavioral regulation in all animals, including insects (Blenau and Baumann, 2001; Blicker and Menzel, 1989; Scheiner et al., 2006; Verlinden et al., 2010). Honeybee societies have emerged as a model insect system for studies of how changes in biogenic amines affect individual behavior (Fussnecker et al., 2006; Lehman et al., 2006; Pankiw and Page, 2003; Sagili et al., 2011; Schulz and Robinson, 2001; Wagener-Hulme et al., 1999). Specific biogenic amines have known roles in the reproductive division of labor between queens and workers (Harris et al., 1996; Wagener-Hulme et al., 1999; Penick et al., 2014), as well as temporal task allocation among workers (Schulz and Robinson, 1999; Wagener-Hulme et al., 1999). Four biogenic amines play significant roles in the honeybee division of labor: dopamine (DA; Agarwal et al., 2011), octopamine (OA; Barron et al., 2007), serotonin (5HT; Harris and Woodring, 1992) and tyramine (TA; Fussnecker et al., 2006; Matsuyama et al., 2015). Each of these amines can have myriad effects on the activities of honey bee workers. For example, OA is positively associated with an increase in overall activity levels (Fussnecker et al., 2006) and the onset of worker foraging (Schulz and Robinson, 2001). Pankiw and Page (2003) found that honey bees treated with OA had significantly lower sucrose response thresholds than controls, a trait that can affect the quantity, quality and type of forage brought back to the colony (Barron et al., 2002; Scheiner et al., 2004). TA concentrations in the brains of nurses and foragers are significantly different, and differentially influence learning behavior (Scheiner et al., 2017). As behaviors of bees have a collective effect on the nest, even small individual differences in the physiological response to the physical and social environment can impact colony welfare.

One group activity critical to honey bee colony function and survival, but still subject to individual behavioral propensities, is thermoregulation (Fahrenholz et al., 1989; Himmer, 1927; Lindauer, 1952). When brood are present, honey bee workers actively maintain the temperature of the hive at 36°C. During the summer, honey bees spread water on the wax honeycombs to evaporatively cool the brood (Kühnholz and Seeley, 1997). Workers also form heat shields by pressing their bodies on comb to absorb heat then remotely disperse the heat (Bonoan et al., 2014; Siegel et al., 2005; Starks and Gilley, 1999). Additionally, workers will fan, flapping their wings while standing at a hive entrance to move hot air out of the colony and allow cool air to flow in (Egley and Breed, 2013; Fahrenholz et al., 1989; Heinrich, 1993). Fanning behavior is of special interest, as it is influenced by individual response thresholds to heat (Jones et al., 2004), social environment (Cook and Breed, 2013) and rates of temperature change (Cook et al., 2016). However, the mechanism regulating these aspects of the fanning response is unknown.

Because of the important roles that biogenic amines play in honey bee task performance, we hypothesized that the inclination to fan was correlated with neurotransmitter concentrations among similarly aged individuals. We compared brain biogenic amine levels between active fanner and non-fanners in identical social conditions. This was done both at hives and in a laboratory setting. We further hypothesized that fanning propensity could be manipulated by treatment with amines found to differ between behavioral phenotypes. To test this, we fed biogenic amines to workers, then measured the fanning response induced by heating. By identifying differences in brain biogenic amines in fanning and non-fanning bees and then using those identified differences to elicit different behavioral responses, we aim to elucidate the proximate mechanisms of the fanning response.

MATERIALS AND METHODS

Honey bees

For comparisons between induced and non-induced fanners and guards, worker bees were collected from 10 European honey bee colonies maintained at the Honey Bee Research Laboratory at the

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Arizona State University Polytechnic Campus in Gilbert, Arizona. For determining the modulatory effects of amines on fanning behavior, European honey bees (*Apis mellifera* Linnaeus 1758) were collected from 11 colonies at the University of Colorado apiary in Boulder, Colorado.

Amine levels in fanning and non-fanning bees

In May 2015, honey bee workers of the fanning caste were collected to determine whether temperature-induced changes in fanning behavior were associated with changes in biogenic amine levels. Fanners were identified as bees standing still near the hive entrance, rapidly moving their wings for at least 10 s, and maintaining an elevated, curved abdomen, and oriented with their heads facing toward the inside of the hive. This is distinct from Nasanov fanning, used to spread a pheromone (Free, 1987), during which the abdomen is straight and the Nasanov gland is exposed at the tip of the abdomen. Fanners at hive entrances were selected because they are more likely engaging in thermoregulation than those workers fanning within the nest to evaporate water from honey. They are relatively easy to identify and collect.

For each trial, two groups of 10 fanners were collected from the same hive by grasping their back legs using forceps, and placing them into a cylindrical (5×2.5 cm) wire mesh containment cage. In groups of 10, honey bees are relatively likely to begin to fan (Cook and Breed, 2013). Fanning is a group response that is almost always initiated all at once, so data were collected only from trials in which all 10 honeybees fanned. Each cage of bees was suspended in the center of a 3.79 liter glass jar (height: 25.65 cm, width: 13.97 cm) placed on a hotplate (Proctor Simplex). The hotplate's coils were lined with aluminum foil to enhance heat distribution. Air temperature adjacent to the cage was measured with a high accuracy digital thermometer (±0.3°C, Cole Parmer).

Once the caged groups of honey bees were placed into heating jars, they were allowed to acclimate for 25 min (Cook and Breed, 2013). After the acclimation period, the ambient start temperature was recorded. For each trial, one group of 'induced' fanners was subjected to a gradual heating at a rate of 1°C min^{-1}, a treatment regime that has been shown to induce fanning behavior (Cook and Breed, 2013; Cook et al., 2016). The second group of each trial, the 'non-induced' fanners, were not subjected to heating. Once honeybees in the heating regime began to fan, cages were removed from both jars and placed directly into liquid nitrogen. The transfer was done as quickly as possible to avoid handling stress-induced changes to amine levels. A total of 21 trials were conducted, but only 19 were used as two induced groups did not fan. Bees were stored intact at −80°C until amine content could be determined. When these samples were run, fanners and non-fanners from the same hive were always paired to help control for inherent hive differences and daily fluctuation in the HPLC system.

Collection of fanners and guards at the hive

To compare the biogenic amine concentrations of honey bees expressing different behaviors at the same hive, we sampled both fanners and guards from the hive porch. Fanners and guards belong to the same age cohort (Egley and Breed, 2013) and are statistically as likely to begin fanning (Cook and Breed, 2013). Fanners were identified as described above, while guards were distinguished by their propensity for splayed wings, actively interacting with incoming bees, or pulling other bees out of the colony (Yang et al., 2010).

Once identified, bees of each caste were grasped with forceps and immediately submerged in liquid nitrogen to snap-freeze them. Mesh was used to compartmentalize both fanners and guards as they were simultaneously collected into the same dewar. We collected 75 guards and 75 fanners. Intact bees were stored at −80°C until amine content could be determined. When these samples were run, fanners and guards from the same hive were always paired to help control for inherent hive differences and daily fluctuation in the HPLC system.

Brain biogenic amine quantification

For each sample, the brains of three bees were pooled to maximize amine detectability. Brain dissections occurred with heads sitting on dry ice to reduce amine degradation. Hypopharyngeal glands and optic lobes were not collected. Brains were placed within a 1.5 ml microcentrifuge tube containing 20 µl of a 0.2 mol l^{-1} perchloric acid solution that also included the internal standards 3,4-dihydroxybenzylamine (DHBA) and synephrine (100,000 pg $µl^{-1}$). Brains were thoroughly homogenized by hand, then sonicated in an ice bath for 5 min. Samples were allowed to sit in the ice bath an additional 15 min to further enhance amine extraction. After extraction, samples were spun in a refrigerated centrifuge (4°C) for 10 min at 12,000 ***g***. Samples were kept on ice and covered until analysis. A maximum of six samples were prepared at a time to reduce the pre-run duration, during which the amines can degrade.

HPLC was used to measure the biogenic amine concentration, as previously described (Brent et al., 2016; Penick et al., 2014). Briefly, brains were homogenized in 20 µl of chilled perchloric acid (0.2 mol l^{-1}) containing DHBA (87 pg $µl^{-1}$; Sigma-Aldrich, St Louis, MO, USA) and synephrine (50 pg $µl^{-1}$; Sigma-Aldrich) as internal standards. The HPLC system (Coularray 5600A, ESA, Chelmsford, MA, USA) was connected to a reverse-phase catecholamine HR-80 column (ESA). A sample volume of 10 µl was injected manually (Rheodyne 9125, Rohnert Park, CA, USA) into a 20 µl holding loop. Samples were passed through a four-channel electrochemical detector, with voltages set at –125, 175, 425 and 650 mV. The detection limit for the HPLC is 0.2 ng. Each amine is detected by each channel, but the specific response elicited by a particular channel voltage will vary depending on the structure of the amine. The net result is a stacking of peaks at the same time, all corresponding to a different channel. The variability across amines in the responses to channel voltage permits discrimination of one amine from another when used in conjunction with peak retention time. The structurally similar amines OA, TA and synephrine produce large peaks at 650 mV and smaller ones at 425 mV. DA, 5HT and DHBA produce large peaks at 425 mV, and successively smaller peaks at 650 and 175 mV. The mobile phase consisted of 15% methanol, 15% acetonitrile, 1.5 mmol l^{-1} sodium dodecyl sulfate, 85 mmol l^{-1} sodium phosphate monobasic, 5 mmol l^{-1} sodium citrate and polished water. The pH was adjusted to 5.6 using phosphoric acid. The mobile phase flow rate was 1 ml min^{-1}. Results are presented as picograms per brain, calculated from curves of external standards (hydrochloride forms of DA, OA, 5-HT and TA; Sigma-Aldrich).

Biogenic amine manipulation of fanning behavior

To test the role of biogenic amines in fanning behavior, honey bees were fed *ad libitum* 2 mol l^{-1} sucrose solutions containing one of several possible treatments. For bees, feeding has been shown to be as effective as injection in elevating circulating neurotransmitter concentrations without the negative effect of physical trauma (Barron et al., 2007). Concentrations of 2 mg ml^{-1} were used to test the effects of OA or TA alone (Barron et al., 2007; Pankiw and Page, 2003; Schulz and Robinson, 2001). A third treatment with 2 mg ml^{-1} of both OA and TA was used to test their combined effect, because

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these amines have been shown to act independently on separate G-protein-coupled receptors (Roeder, 2005). Sucrose alone was used as a control. New solutions were made daily, and no more than three trials were run with the same solution.

Solutions were blind labeled before being assigned to treatment groups. Test groups were composed of five actively fanning bees randomly selected from a hive entrance. Fanners were held by their hind legs and a 10 µl aliquot of test solution was presented by pipette to the antennae of the honeybee until she extended her proboscis and drank the entire droplet. If the honeybee did not drink within 30 s, or if she did not drink the entire droplet, she was released and another fanner was chosen.

We placed fed bees in color-coded cages that matched the blind labeling of the treatment vial for that day. For one round of all five treatments, all bees were collected from the same hive to control for inherent hive differences. After feeding, caged honey bees were placed in a heating chamber (described above), where they were allowed to acclimate for 20 min. Because biogenic amines take 30–40 min to influence behavior but degrade quickly afterwards (Fussnecker et al., 2006), the feeding and acclimation times were optimized to detect induced changes, as the typical protocol is a 25 min acclimation. Bees were subjected to the heating protocol and behavioral assay as described above, and the incidences of fanning and the temperatures at which fanning occurred were recorded. All treatments were conducted between July and September 2015.

Statistical analysis

Because biogenic amine concentrations were found to be non-normally distributed, we used a nested ranks test to compare between induced fanners and non-induced fanners, and between fanners and guards. Nested ranks tests allow us to perform a Mann–Whitney rank sum test while treating hive as a random effect. We treated hive as a random effect to control for potential natural variation across hives (Harris and Woodring, 1992). All analyses were performed on per-brain concentrations. We then $\log_{10}$-transformed amine data to more effectively graph the values, as there was a large spread between DA and the rest of the biogenic amines.

To analyze the effect of biogenic amine feeding treatments, the proportion of fanners in any given trial was treated as a two-column response variable (number of fanners, number of non-fanners). We also treated hive as a random effect to control for variation across hives. We then used this response variable in a logistic regression, performed with the glmer function in R. We performed an analysis of deviance test against a null model for overall characterization of our model.

Statistical comparisons were made using the base package for R (version 3.0.2), and graphs were created with ggplot2 (version 2.1.0).

RESULTS

HPLC analysis of induced versus non-induced fanners

A comparison of the whole-brain concentration of four neurotransmitters (Fig. 1) indicated that active fanners had significantly lower concentrations of OA (nested rank test: Z=0.4, n=39, P=0.029) and TA (Z=0.622, n=39, P=0.002) than non-induced bees. There was no difference between induced fanners and non-induced fanners for DA (Z=0.2, n=39, P=0.187) or 5HT (Z=0.144, n=39, P=0.234).

Treatment with biogenic amines

To test for a causal relationship between fanning behavior and levels of OA and TA, identified fanners were fed one or both of these biogenic amines, or a sucrose control. Treatment responses varied significantly across treatments (n=104, χ^2_3=13.86, P=0.003; Fig. 2). Honey bees treated with both OA and TA were significantly less likely to begin fanning than those given the sucrose control (logistic regression: n=52, effect size=−0.79, Z=−2.966, P=0.003) or OA alone (n=52, effect size=−0.82, Z=3.09, P=0.002). Additionally, TA-treated bees were significantly less likely to fan than those treated with OA (n=52, effect size=−0.523, Z=2.033, P=0.0421). There was no difference between those treated with TA or those fed both amines (n=52, effect size=0.298, Z=1.089, P=0.276). Finally, fanning likelihood relative to that for bees fed the sucrose control did not differ for those fed either just OA (n=52, effect size=0.031, Z=−0.123, P=0.90) or TA (n=52, effect size=0.49, Z=1.91, P=0.0561), although TA seems to have a stronger, although insignificant, inhibitory effect than OA.

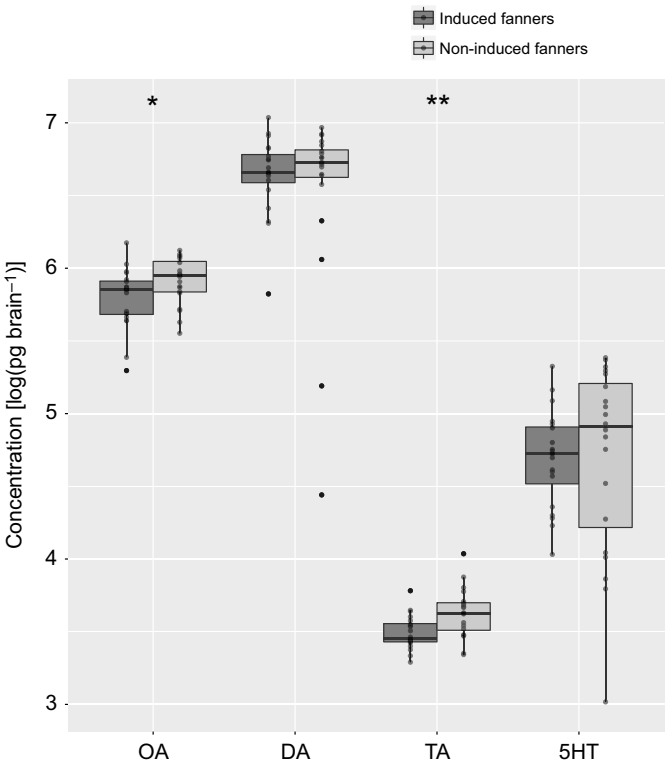

Fig. 1. Concentrations of four biogenic amines in induced fanners compared with non-fanning honeybees. Active fanners had significantly lower concentrations of octopamine (OA; nested rank test: Z=0.4, n=39, P=0.029) and tyramine (TA; Z=0.622, n=39, P=0.002) than non-induced bees. Concentration is $\log_{10}$-transformed. Asterisks indicate level of significance (*P>0.05, **P>0.005). Horizontal bars are medians, boxes are 25th–75th percentiles, lines are 1.5× interquartile range (IQR), points are Tukey outliers. N=39, with three brains pooled per sample.

Biogenic amines of fanners and guards

To test the hypothesis that similarly aged but behaviorally different guards and fanners exhibit differences in biogenic amine levels, we again measured whole-brain concentrations. There were no significant differences between guards and fanners for any of the measured amines (nested rank test: OA, Z=0.171 n=48, P=0.205; DA, Z=−0.184, n=48, P=0.85; TA, Z=0.144, n=48, P=0.244; 5HT, Z=−0.157, n=48, P=0.813; Fig. 3).

DISCUSSION

Here we report that OA and TA modulate thermoregulatory fanning. Honey bees induced to fan in the laboratory by heat application had

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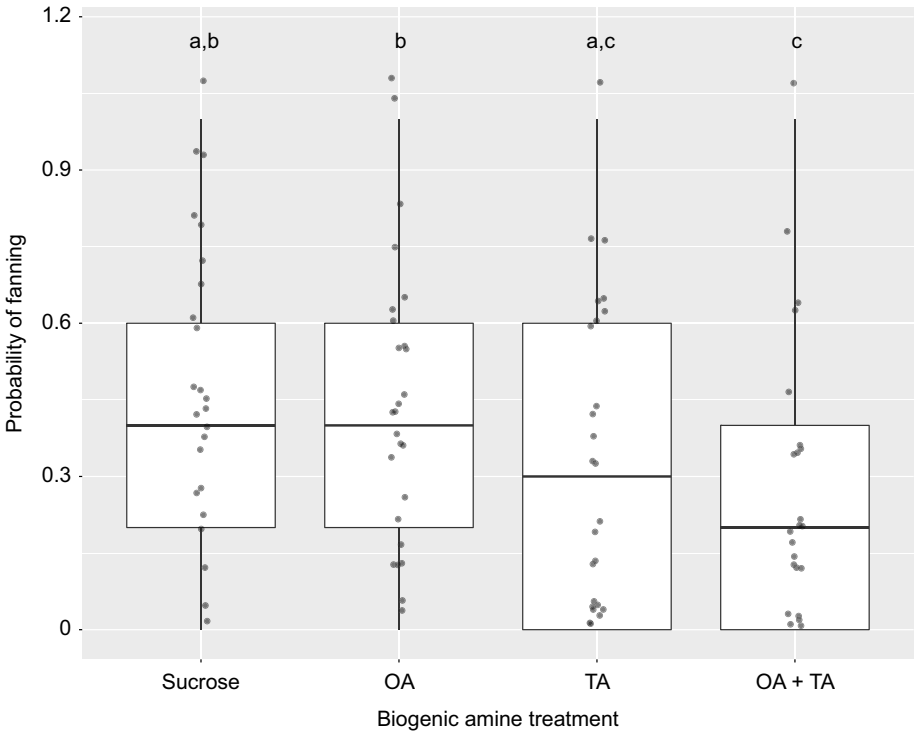

Fig. 2. Frequency of fanning in response to biogenic amine treatments. Only bees treated with OA and TA together fanned significantly less often than controls fed only sucrose (analysis of deviance against null model: n=104, χ^2_3=13.86, P=0.003). Different lowercase letters indicate a significant difference between treatment groups at α=0.05. Horizontal bars are medians, boxes are 25–75th percentile, lines are 1.5×IQR, points are Tukey outliers. Test groups consisted of five honey bees.

lower concentrations of both of these biogenic amines than controls (Fig. 1), and bees fed a combination of the two biogenic amines exhibited a reduction in fanning behavior (Fig. 2). When administered individually, neither of the amines induced a significant reduction of fanning, suggesting that the two may act synergistically to modulate the bees' response to rising temperature.

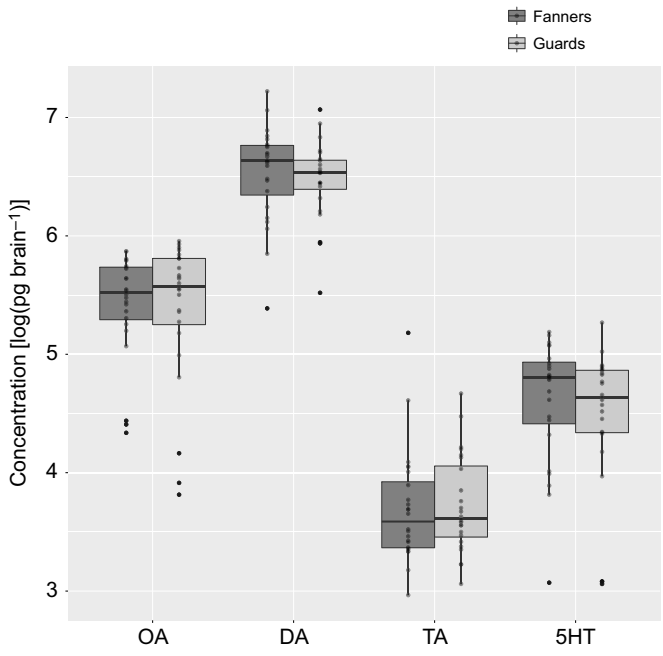

Fig. 3. Concentrations of four biogenic amines in fanners and guards collected at the hive. Concentration is $\log_{10}$-transformed. There were no significant differences between these groups for any of the amines, as compared by a nested rank test. Horizontal bars are medians, boxes are 25th–75th percentiles, lines are 1.5×IQR, points are Tukey outliers. N=24 for both fanners and guards, with three brains pooled per sample.

Contrary to our predictions, guards and fanners have similar brain biogenic amine concentrations. However, there is evidence that guards and fanners may switch between these tasks over days (Egley and Breed, 2013), as well as over hours (C.N.C., J. Ternest and M.D.B., unpublished data). Furthermore, the typical age of a guard (Wagener-Hulme et al., 1999) is within the age range (14.7 to 19 days) of workers that perform nest ventilation (Seeley, 1982). Wagener-Hulme et al. (1999) showed that there is no significant difference in DA, OA or 5HT among bees within this age range. While our data show that heating bees can induce changes in amines that increase the likelihood of fanning, our comparison between guards and fanners indicate that the behavior is not solely regulated by amine level.

Despite being collected simultaneously from the same hot environment at the hive entrance, having similar amine profiles (Fig. 3) and being just as likely to fan when heated sufficiently in the laboratory (Cook and Breed, 2013; Egley and Breed, 2013), guards did not exhibit fanning when in similar environmental conditions and instead maintained defensive postures. These results suggest that, in addition to a heat-induced reduction to amine concentration, the propensity for fanning could be regulated by a specific amine threshold that varies by behavioral caste. In future studies, we will pharmacologically manipulate guards to determine whether fanning is equally diminished among that task group. The threshold would appear to be higher for guards than for fanners so that evoking the same behavioral response would likely require the environment to be substantially hotter. Another possible driver of behavioral differences is the ratios of biogenic amines, which might produce interactive effects (Seid and Traniello, 2005); however, we found that the ratios in guards and fanners were similar (see Appendix). While the thermal threshold needed to induce a certain level of biogenic amine expression was statistically similar for both guards and fanners, the differences in thermal response threshold may be due individual variation in OA and TA receptor expression.

Fanning is behaviorally similar to foraging. Fanning is influenced by thermal response thresholds (Jones et al., 2004), and is

modulated by social environment (Cook and Breed, 2013). It is also likely similar in metabolic need, given the active wing movement that defines fanning. Individual foraging propensity in honey bees also appears to be influenced by multiple mechanisms, including response to sucrose receptor expression (Hunt et al., 1995; Page et al., 1998), OA levels (Pankiw and Page, 2003) and OA receptor expression (Reim and Scheiner, 2014). Foragers have elevated TA titers in their brains, compared with nurses, and show increased appetitive learning. TA treatment also increases the gustatory and learning responses (Scheiner et al., 2017). TA is a precursor to OA (Roeder, 2005), although they act individually on independent receptor proteins. They are thought to be analogous to noradrenaline and adrenaline, respectively. OA plays a role in in insect stress and flight, enhancing muscle performance (Malamud et al., 1988) and increasing glycolysis (Goosey and Candy, 1982). Furthermore, exposure to high temperatures may be eliciting a stress response in the bees. Using agonists and antagonists to TA and OA, as well as using antibodies to identify where the receptors for these biogenic amines are, will illuminate the neuropathway that controls the fanning response.

Our findings contribute to the growing field of socioneuroethology (Kamhi and Traniello, 2013). Research in several ant species has found that biogenic amines affect individual response thresholds that can then affect group behavior. For example, Muscedere et al. (2012) found that *Pheidole dentata* workers with pharmacologically lower 5HT levels displayed reduced sensitivity to trail pheromone. Kamhi et al. (2015) found that OA affected aggressive behaviors in both major and minor *Oecophylla smaragdina* workers. Both of these modulated behaviors can have dramatic effects on labor across the colony, with foraging behavior and nest protection, respectively, being affected. Similarly, our previous work shows that fanning behavior is a socially modulated behavior that is affected by group size (Cook and Breed, 2013). It is also influenced by how quickly the thermal environment changes, with larger groups fanning both at earlier temperatures and with more individuals fanning, when heated at a faster rate (Cook et al., 2016). This cooperative group behavior likely emerges from individuals using proximate social and environmental information to coordinate a response (Camazine et al., 2001). An individual's ability to sense or process that information through biogenic amines means that there are downstream and potentially non-linear consequences for coordinated group behavior.

While we did not find a significant effect of biogenic amines on the thermal response thresholds for fanning, we have shown that the likelihood of fanning is decreased by treatment with both OA and TA. However, when honeybees were fed both OA and TA, they were less likely to begin to fan, especially as we observed normal activity across all groups. Even if one bee was still likely to begin to fan, it did not set the other bees fanning, as typically happened in the untreated groups. Fanning is most effective when multiple bees are performing the job; therefore, this alteration of the group response by modulating biogenic amines may have implications for the temperature regulation of the hive as a whole. By exploring the relationship between physiological mechanisms and performance of behavior, we help to provide the framework by which to explore how individual variation among many workers optimizes task allocation and the division of labor in eusocial insects.

APPENDIX

We evaluated the ratios of amines to explore how they could be potentially working synergistically. Because this was a *post hoc* analysis, we feel that few conclusions can be drawn from this information. Responses to varied ratios were not directly tested, and we do not know how dopamine and TA interact to influence behavior. Exploring the relationship of the amines, and how they may influence or be influenced by receptor expression, would require a substantially different approach than we took with this research. We do, however, believe it is important to share these analyses.

Guards versus fanners

We found no significant differences across the ratios of measured amines (Mann–Whitney rank sum test: OA:DA, W=362, P=0.131; OA:TA, W=271, P=0.736; OA:5HT, W=325, P=0.455; DA:TA, W=238, P=0.31; 5HT:DA, W=253, P=0.480; 5HT:TA, W=253, P=0.4801).

Induced versus non-induced fanners

We found that the proportion of DA:TA was significantly different between induced and non-induced fanners (Mann–Whitney rank sum test: W=297, P=0.008). There was no significant difference in any of the amine ratios (Mann–Whitney rank sum test: OA:DA, W=153, P=0.211; OA:TA, W=248, P=0.199; OA:5HT, W=194, P=0.883; 5HT:DA, W=160, P=0.289; 5HT:TA, W=237, P=0.327).

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Competing interests

The authors declare no competing or financial interests.

Author contributions

C.N.C., C.S.B. and M.D.B. conceived of the study and drafted the manuscript. C.N.C. and C.S.B. conducted the experiments.

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Data availability

Data are available online on Figshare: https://figshare.com/s/3e0f099ac2a3042a1a12, https://figshare.com/s/411738422b6c325278f4, https://figshare.com/s/6ac068951f4f0143ca5c

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