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Octopamine affects gustatory responsiveness and may enhance learning in bumble bees

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Abstract – Octopamine has broad roles within invertebrate nervous systems as a neurohormone, neurotransmitter, and neuromodulator. It orchestrates foraging behavior in many insect taxa via effects on feeding, gustatory responsiveness, and appetitive learning. Knowledge of how this biogenic amine regulates bee physiology and behavior is based largely on study of a single species, the honey bee, *Apis mellifera*. Until recently, its role in the foraging ecology and social organization of diverse bee taxa had been unexplored. Bumble bees (*Bombus* spp.) are a model for research into the neural basis of foraging and learning, but whether octopamine similarly affects sensory and cognitive performance in this genus is not known. To address this gap, we explored the effects of octopamine on gustatory responsiveness and associative learning in *Bombus impatiens* via conditioning of the proboscis extension reflex (PER) using a visual (color) cue. We found that octopamine had similar effects on bumble bee behavior as previously reported in honey bees; however, higher doses were required to induce these effects. At this higher dose, octopamine lowered bees' gustatory responsiveness and appeared to enhance associative learning performance during the early phase of our experiment. Adding to recent studies on stingless bees (*Meliponini*), these findings support the idea that octopamine's role in reward perception and processing is broadly conserved across Apidae, while pointing towards some differences across systems worth exploring further.

biogenic amines / foraging / sensory processing / *Bombus impatiens* / bees / color

1. INTRODUCTION

Octopamine (OA) is a biogenic amine involved in a diverse suite of physiological processes in insects (Roeder 1994, 1999). In honey bees (*Apis mellifera*), it may influence phenomena as diverse as circadian and cardiac rhythms (Bloch and Meshi 2007; Papaefthimiou and Theophilidis 2011), the stress response (Harris and Woodring 1992), and motor performance (Fussnecker et al.

2006). However, its clearest role is in the nervous system where it mediates sensory and cognitive processes associated with feeding (Giurfa 2006; Rein et al. 2013). Alongside other biogenic amines (e.g., dopamine (DA) and tyramine (TA), OA's precursor), OA has well-established effects on sensory responsiveness (Barron et al. 2002; Scheiner et al. 2014; Schilcher et al. 2021), including responsiveness to sucrose (Pankiw and Page 2003; Scheiner et al. 2002). These effects on gustatory responsiveness are in turn a key determinant of learning performance in a foraging context (Scheiner et al. 2001). OA is centrally involved in the reward pathways that underlie appetitive learning: its injection into brain regions involved in learning and memory substitutes for

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a reward in a PER (proboscis extension reflex) conditioning paradigm (Hammer and Menzel 1998; Riemensperger et al. 2005; Schwaerzel et al. 2003; Unoki et al. 2005). OA’s heightened presence in the brains of starved foragers suggests that it also helps regulate the appetite—and perhaps more broadly, the motivation to learn—of workers in a feeding context (Mayack et al. 2019, see also Akülkü et al. 2021).

These effects of OA on individual *A. mellifera* behavior may scale up to influence the division of labor and collective foraging efforts more generally (Wagener-Hulme et al. 1999). In the brains of nurses vs. foragers, OA receptor expression differs (Reim and Scheiner 2014; Schulz and Robinson 2001), as do OA titers (Schulz et al. 2002). Among foragers, patterns of OA receptor expression change with age (Peng et al. 2021), and OA-mediated differences may underlie individual-level patterns of resource specialization (Arenas et al. 2021; Giray et al. 2007). For example, OA’s influence on sucrose response thresholds determines the quality of food they bring back when foraging (Giray et al. 2007; Pankiw and Page 1999). Pollen foragers have lower sucrose response thresholds and as such are less discriminating in the nectar they will accept compared to nectar foragers (Page Jr et al. 1998; Scheiner et al. 2001). OA also mediates social transmission of information about food resources: for example, bees treated with

OA over-represent the quality of the forage they encounter when communicating with nestmates via their “dance language” (Barron et al. 2007a). Interestingly, OA affects dances for both pollen and nectar quality in the same way, indicating that it plays a role in reward processing more broadly and thus has a role equivalent to the dopaminergic system in mammals (Wise 2004).

Given how clearly OA is involved in the regulation of individual and colony-level foraging behavior in *A. mellifera*, what role does it play for other bees? A 2022 Web of Science search of the scientific literature for “octopamine + bee” confirmed that while honey bees have historically offered a tractable model for untangling complex relationships between aminergic systems, individual physiology, and collective behavior, other bee taxa are rarely considered (Figure 1). Perhaps this reflects the assumption that OA’s key roles in these sensory and neural processes are so fundamental that they must be broadly conserved, though recent reviews highlight the need for more information across species (rev. Kamhi et al. 2017; Sasaki et al. 2021) that could help test this assumption. Indeed, a recent study of the closely related TA signaling system pointed towards a shared neural expression of TA receptors among representatives of Apini, Bombini, Meliponini, and Osmiini (Thamm et al. 2021), although behavioral data is needed to confirm if similar expression patterns relate to similar

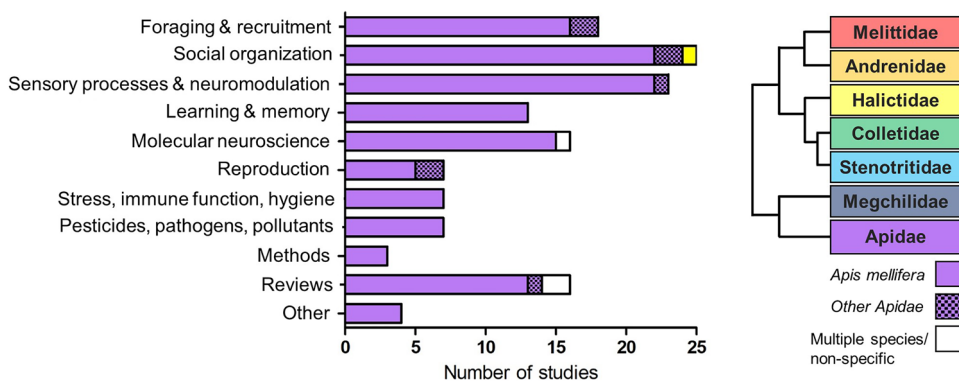


Figure 1. Summary of studies from a 2022 Web of Science search of the scientific literature for “octopamine + bee.” Color indicates bee family; Apidae and specifically *Apis mellifera* are greatly over-represented in the literature compared to other bee families.

functionality. Likewise, behavioral work on stingless bees points to a conserved effect of OA on sucrose responsiveness and foraging behavior: *Melipona scutellaris* fed OA had a lower sucrose response threshold (Mc Cabe et al. 2017) and *Plebeia droryana* foraged on a sucrose feeder containing OA at a faster rate compared to their behavior at a control feeder (Peng et al. 2020).

On the other hand, recent comparative work has also revealed intriguing potential for differences in aminergic pathways. Thamm et al.'s (2021) study noted genus-level differences in the expression patterns of a tyramine receptor (AmTAR1) within the optic lobes. Likewise, within honey bees, OA receptor SNPs were associated with different ecotypes raising the prospect of their role in adaptation to elevation-specific foraging ecologies (Wallberg et al. 2017). Given variation in bee sociality, dietary specialization, and life histories (often involving both social and solitary foraging phases), exploring whether the behavioral effects of OA that are most established in *A. mellifera* manifest in other species will help fill in the picture of how this appetitive system supports diverse foraging behaviors across the bee tree of life.

Bumble bees (*Bombus*) are an important model for the study of insect cognition and foraging behavior (Chittka and Thomson 2001). Like *Apis*, *Bombus* are generalist foragers that visit a variety of flowers when foraging, and as such must rapidly discriminate between floral rewards (e.g., nectars differing in sucrose concentration) and learn which flowers contain the highest quality rewards based on associated floral stimuli (color, scent, etc.). Typically living as part of a colony, bumble bees communicate information about resource availability, albeit through chemical communication rather than a waggle dance (Dornhaus et al. 2003). Despite these shared features, bumble bees show a number of cognitive (Sherry and Strang 2015) and neural (Gowda and Gronenberg 2019) differences from honey bees. Given that individual *Bombus* workers are less specialized in their roles within the colony than in *Apis* and in their collection of resources more generally (Goulson 2003), OA's role in coordinating foraging-related behaviors is an open question.

Here we addressed the role of OA in bumble bee sensory responsiveness and cognition. Following a protocol similar to those used in the past with honey bees (Pankiw and Page 2003; Scheiner et al. 2002) and stingless bees (*Melipona scutellaris*; Mc Cabe et al. 2017), we addressed how OA affected gustatory responsiveness and learning of a visual association in bumble bees *B. impatiens*. If OA has a similar role in bumble bees as it does in honey bees and stingless bees, then we expected its ingestion to increase gustatory responsiveness and enhance appetitive learning in a dose-dependent manner.

2. METHODS

2.1. General methods

In all experiments, we used *Bombus impatiens* workers (Experiment 1 $n=65$; Experiment 2 $n=56$) purchased from Koppert Biological Systems (Howell, MI, USA). Bumble bee colonies were maintained indoors at the University of Nevada, Reno. To obtain individuals for testing, we used an insect aspirator to remove bees from wicked feeders (Exp. 1: 30% (w/w) sucrose; Exp. 2: 15% (w/w) sucrose) in a central foraging arena (L×W×H: 100×95×90 cm) which had 3–5 colonies attached at any one time. We supplemented colonies with 5 g of honey bee pollen (Koppert Biological Systems, Howell, MI, USA) every 2 to 3 days.

Following Riveros and Gronenberg (2009) and Riveros et al. (2020), we cooled bees in plastic vials placed on ice to immobilize them. Bees were then placed into individual plastic tubes (modified 1000- μ l pipette tips, Figure 2a) and restrained with two metal insect pins forming a “yoke” between their head and thorax that was secured with tape to the plastic tube (as in Muth et al. 2015; Riveros and Gronenberg 2009). The bee could extend its proboscis and move its antennae but was otherwise immobilized. Bees were left to acclimate for 3 h at room temperature in a dark room. After this time, we screened bees for responsiveness by presenting a droplet of 30%

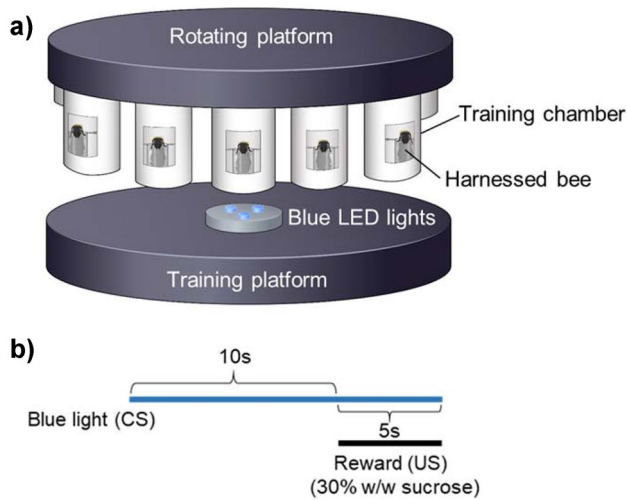


Figure 2. A diagram of the proboscis extension response (PER). **a** Training apparatus and **b** training protocol used in Experiment 2.

(w/w) sucrose to their antennae; bees that did not exhibit PER were removed from the experiment.

All experiments were conducted in a dark room, illuminated only with a red light to reduce any additional visual stimuli that could influence responsiveness or learning. In all experiments, we fed bees OA, rather than injecting or exposing bees topically with the OA dissolved in a solvent. All three methods have been used in the past, and OA can reach all tissues (including the brain) via all methods (Barron et al. 2007b). We chose oral treatment since it is non-invasive and has been an effective method in the past (Pankiw and Page 2003; Scheiner et al. 2002).

All statistical analyses were performed in R version 4.1.2 (2021) (R Core Team 2020). We carried out GLMMs using the `glmer` function in the `lme4` package (Bates et al. 2015), including “bee” as a random factor to control for the multiple measures per bee. To determine the significance of interaction effects, we ran models with and without the interactions and used the `anova()` function to compare the fit of models using AICs. We carried out post-hoc tests using the `emmeans` package (Lenth 2017) and visualized relationships using `effects()` (Fox and Weisberg 2019).

2.1.1. Experiment 1: Does OA affect gustatory responsiveness in bumble bees?

To determine whether OA affected gustatory responsiveness, we assigned bees randomly to one of three treatments that varied in the solution they were fed prior to testing. In all treatments, we used a Hamilton syringe to feed bees 10 μl of 30% (w/w) sucrose containing (1) 0 $\mu\text{g}/\mu\text{l}$ OA (control), (2) 2 $\mu\text{g}/\mu\text{l}$ OA (0.013 M) (i.e., a total of 20 μg), or (3) 8 $\mu\text{g}/\mu\text{l}$ OA (0.052 M) (i.e., a total of 80 μg). Final sample sizes were $n=23$, 27, and 25 for control, 2 $\mu\text{g}/\mu\text{l}$ OA, and 8 $\mu\text{g}/\mu\text{l}$ doses, respectively. After feeding bees, we allowed them to sit for 30 min to allow full absorption of the OA (Pankiw and Page 2003). All three treatments were represented on a given day.

We tested the gustatory responsiveness of all bees by presenting them with eight different concentrations (w/w) of sucrose solution in succession (0.01%, 0.03%, 0.1%, 0.3%, 1%, 3%, 10%, 30%, 50%), with a presentation of water at the beginning and between each sucrose presentation (as in McCabe et al. 2017; Pankiw and Page 2003). As in these previous studies, presentation of water allowed us to distinguish a

possible increase in sucrose responsiveness from a generalized increase in responsiveness (e.g., to other gustatory or chemotactile stimuli sensed by antennae). For each water trial, we presented the liquid to the bees' antennae and allowed them 3 s to respond, before presenting them with the sucrose solution, and again giving them 3 s to respond. The inter-trial interval between each sucrose presentation was 5 min.

2.1.2. Experiment 1 data analysis

To determine whether bees assigned to the three pre-treatments differed in their responsiveness to sucrose, we carried out a binomial GLMM with the binary response variable of whether the bee responded or not (1/0) and the following explanatory variables: sucrose concentration (continuous), treatment (3 levels), and the random factor "bee." We initially planned to use a similar model to compare responsiveness to water, but due to the large number of bees that did not respond to this stimulus, we just compared the first water trial (during which we observed the greatest response) using a binomial linear model with the response variable "1" responded or "0" did not respond.

2.1.3. Experiment 2: Does OA affect visual learning in bumble bees?

We trained bumble bees via a visual conditioning paradigm similar to one used previously with bumble bees (Riveros and Gronenberg 2012). We harnessed 56 bees and trained and tested them using the proboscis extension response (PER) protocol. Bees were randomly assigned to two treatments and fed prior to training 10 μl of 30% (w/w) sucrose containing either (1) 0 $\mu\text{g}/\mu\text{l}$ OA (control; $n=28$) or (2) 8 $\mu\text{g}/\mu\text{l}$ OA (treatment; $n=28$). This dose was informed by our findings from Experiment 1. After being fed, individuals were transferred to the PER training apparatus and left to sit for 30 min before undergoing training and testing. Bees from both treatment groups were represented equally on each testing day.

The PER training apparatus consisted of a circular rotating platform suspended above the tabletop (Figure 2a). Twelve "training chambers" created from plastic cylinders were glued to the underside of this platform, approx. 6 cm apart. An opening (w \times h: 3 cm \times 1.5 cm) in each training chamber allowed experimental access to the harnessed bee. Apart from a thin platform supporting the harnessed bee, the underside of each training chamber was open, allowing light to enter in from below (on which three blue ($\lambda=470$ nm) LED lights were mounted). Each chamber was lined with aluminum foil to evenly disperse lights which were controlled via a switchboard.

In an absolute conditioning paradigm, each bee was given 11 training trials followed by a test trial. While our previous work using absolute conditioning to train bees to the same blue LED conditioned stimulus (CS+) as used here (Muth et al. 2018; Riveros et al. 2020) involved 8 trials, in that previous work we observed ~40–60% of subjects showing a conditioned response at the last trial; we thus increased the number of trials here to 11 in order to give bees a greater opportunity to learn. Each training trial consisted of a presentation of the CS+ (blue light), followed by the unconditioned stimulus (30% (w/w) sucrose). In the initial trials, we exposed a bee to the CS+ for 10 s before presenting the bee with the sucrose reward for an additional 5 s (2 s to antennae, 3 s to proboscis) (Figure 2b). After the bee showed a conditioned response, the reward was presented (for 3 s) as soon as the bee extended its proboscis (even if 10 s had not elapsed). In all cases, the reward and stimulus were removed simultaneously. As in Exp. 1, we used an inter-trial interval of 5 min. The test trial was the same as the training trials with the exception that the CS+ was given without the reward. While in all trials, bees were not given a sucrose reward until after they extended their proboscis, unrewarding test (or probe) trials are typical in learning assays to ensure that an animals' response is to the conditioned stimulus rather than the unconditioned stimulus or another feature associated with it. In all learning and test trials, we recorded (via live observation) whether the individual bee extended its proboscis in response to the blue light, and in

cases when they did not but were presented with a reward (i.e., during the learning trials), if they responded to the presentation of the reward. This allowed us to not only determine if learning performance differed between the treatment groups but also if overall tendency to respond to sucrose presentation also differed.

2.1.4. Experiment 2 data analysis

If a bee did not exhibit a proboscis extension to presentation of the sucrose reward more than 4 times across the 11 training trials, then we considered it to be unresponsive and excluded it from further analysis (OA $n=1$; control $n=5$), resulting in final sample sizes of OA $n=27$ and control $n=23$. To analyze whether bees learned differently across trials on the basis of treatment, we carried out binomial GLMMs where the response variable was whether the bee responded to the light stimulus or not (0/1) prior to receiving a reward, and the explanatory variables included were trial, treatment, and the random factor bee. Because both groups showed evidence of learning initially but then a decline after trial 6, we split the data into two models: trials 1–6 and trials 7–11. The test trial data were analyzed alone using a binomial GLM.

To address whether feeding motivation/responsiveness varied across trials, we also carried out models, this time using all 56 bees tested. We included the response variable of whether the bee responded to the sucrose or not once it was presented to them (0/1) and the same explanatory variables as above. Interactions between trial and treatment were always included initially, but excluded if non-significant.

3. RESULTS

3.1. Experiment 1: Does OA affect gustatory responsiveness?

Bees that were pre-fed the higher dose of OA were more responsive to sucrose than both the control and lower-dose treatment, which did not

differ to each other (comparison of models with and without treatment \times concentration interaction: $\chi^2_2=6.830$; $p=0.033$; conditional R^2 of final model = 0.673; Tukey post-hoc comparison between treatments: control vs. low: $z=0.761$, $p=0.727$; control vs. high: $z=4.713$, $p<0.0001$; low vs. high: $z=-4.302$; $p=0.0001$; Figure 3a).

Similarly, in the first water trial, bees assigned to the high-dose pre-treatment were more responsive than the control group ($z=2.408$, $p=0.016$; Figure 3b; conditional R^2 of final model = 0.161), while the bees that were pre-fed the lower dose of OA did not differ from the control bees ($z=0.103$; 0.918; Figure 3b). After the first water trial, bees across all treatments rarely responded at all.

3.2. Experiment 2: Does OA affect visual learning in bumble bees?

3.2.1. Learning performance—response to the conditioned stimulus

Across the first 6 learning trials, performance improved in both bees pre-treated with OA as well as in control bees ($z=4.731$, $p<0.0001$), but the OA-treated bees showed higher performance ($z=-2.196$, $p=0.028$; conditional R^2 of final model = 0.299). From the 7th to 11th learning trial, performance declined in both groups, and there was an interactive effect, where the OA-treated bees at first out-performed the control group, but this effect disappeared towards the end of training (treatment \times trial: $z=2.021$; $p=0.043$; trial $z=-2.781$; $p=0.005$; treatment: $z=-2.205$, $p=0.027$; conditional R^2 of final model = 0.341; Figure 4a). There was no effect of treatment in the test phase ($z=0.167$; $p=0.867$; conditional R^2 of final model = 0.001); however, overall response was very low by this point (Figure 4a).

3.2.2. Responsiveness—response to the unconditioned stimulus

To address whether bees' motivation to respond to the unconditioned stimulus (sucrose reward) varied across treatments, we compared

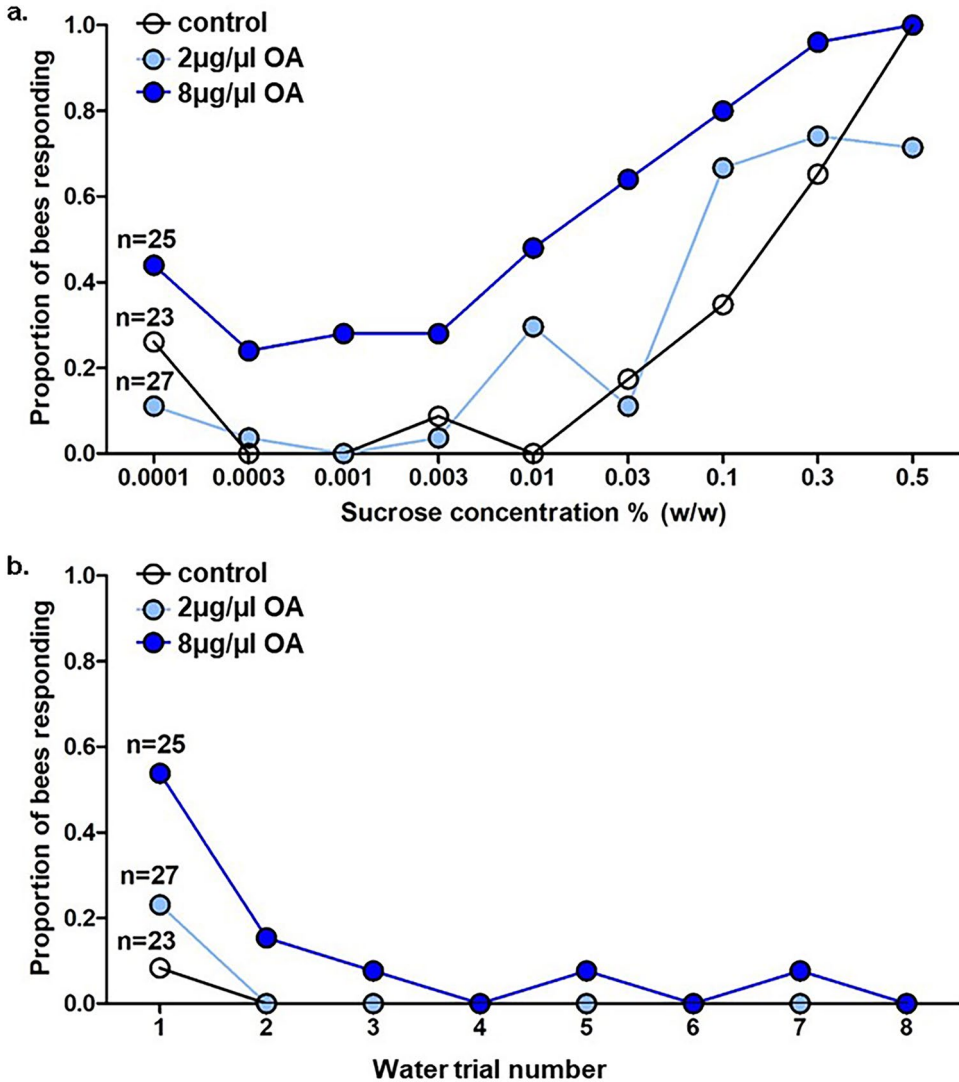


Figure 3. OA effects on bumble bee sucrose responsiveness (Experiment 1). When bees were pre-fed OA of two doses, **a** sucrose responsiveness increased at the higher, but not lower, dose, and **b** initial responsiveness to water was higher in the high OA-treated group.

whether bees in the OA-treated and control groups responded similarly once the sucrose reward was presented to them. Our results suggest that initially the motivation to feed dropped in the control treatment but remained in the OA treatment; however, towards the end of the training period, bees assigned to both treatments showed similarly low motivation to consume the sucrose reward (treatment × trial: $z = 2.444$; $p = 0.015$; trial $z = -4.347$;

$p < 0.001$; treatment: $z = -3.604$, $p < 0.001$; conditional R^2 of final model = 0.428; Figure 4b).

4. DISCUSSION

Octopamine (OA) has long been known to play an important role in orchestrating the foraging behavior of honey bees (rev. Giurfa 2006;

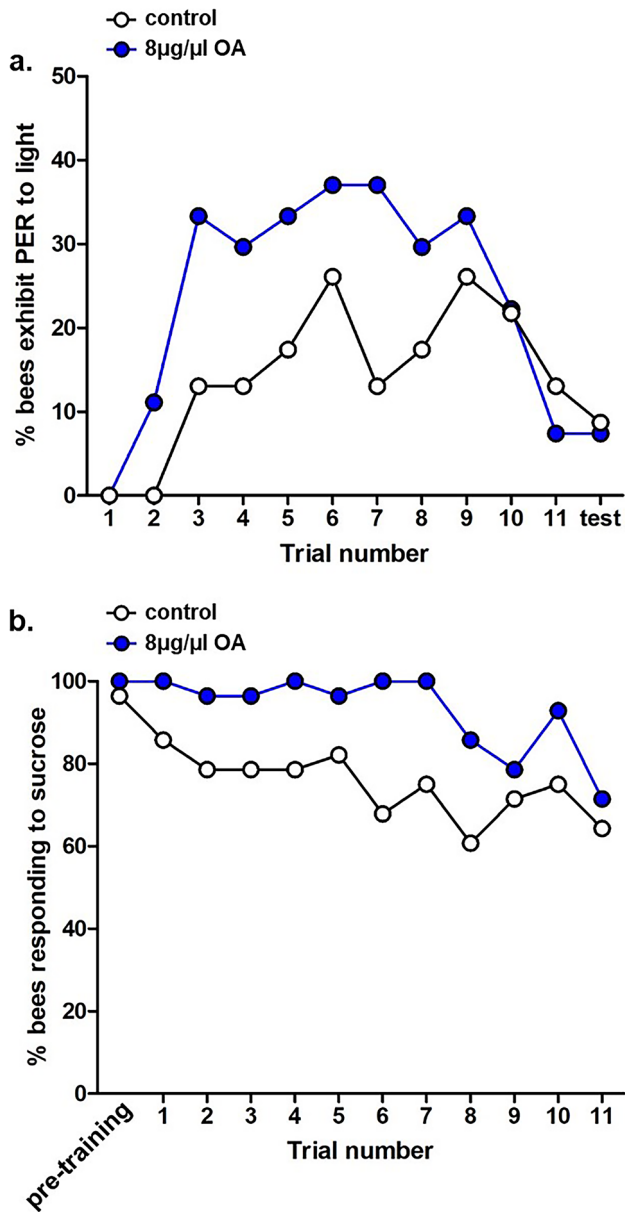


Figure 4. OA effects on bumble bee learning (Experiment 2). **a** Bumble bees pre-fed a high dose of OA were more responsive to the conditioned stimulus than a control group; dashed line indicates where motivation to respond dropped across both treatments. **b** The proportion of bees responding to the sucrose reward was higher in the OA-fed group than the control group.

Roeder 1999), a system often used as a model to study the neural basis of bee behavior (Menzel 2012) and the physiological mechanisms of task specialization (Riveros and Gronenberg 2010).

Yet, how OA affects behavior and physiology in other bee taxa exhibiting different levels of sociality (e.g., Halictidae: Jeanson et al. 2008; Smith et al. 2019; *Ceratina*: Cook et al. 2019)

is only beginning to be explored (Figure 1). Our understanding of how OA mediates collective foraging in other social bees (e.g., Meliponinae; Mc Cabe et al. 2017; Peng et al. 2020) is equally limited. Within *Bombus*, only five prior studies have, to our knowledge, directly measured or manipulated OA. Four of these involve measuring OA levels or related gene expression with the aim of understanding reproductive division of labor: Bloch et al. (2000) found that OA titers in *Bombus terrestris* correlated with the dominance status of workers, independent of age or ovarian development; more recently, Sasaki et al. measured OA levels in *Bombus ignitus* queens at different reproductive stages (Sasaki et al. 2017) or across workers vs. queens (Sasaki et al. 2021). Besides the present study, the only other experiment on *Bombus* that considers OA's role in a foraging context appears to be Cnaani et al. (2003), which asked whether OA altered floral choice in *B. impatiens*. This experiment used a free-flying assay with automatically refilling artificial flowers to show that the presence of OA in "nectar" impacted *B. impatiens* workers' persistence visiting a food source that became unrewarding. Although these results have intriguing implications for understanding how nectar chemistry might activate octopaminergic pathways (Muth et al. 2022), this experiment was not designed to identify the mechanism behind shifts in floral choice. Indeed, understanding how OA (or other biogenic amines) influences foraging behavior in diverse bee taxa will require standardized and easily replicable behavioral assays. To this end, we adapted two protocols that have long been widely used to study the effects of OA on honey bee (and recently, stingless bee) learning. Using these, we found that OA has an analogous effect on bumble bees as in these two other genera, increasing gustatory responsiveness and seeming to enhance associative learning. Our results indicate that similar mechanisms may underlie appetitive learning within Apidae, but also highlight differences that may inform future work in this and other systems.

Our first experiment explored how consumption of OA at two concentrations affected bees' responsiveness to water and sucrose solutions.

Broadly in keeping with work on honey bees, we report the first evidence that OA consumption increases gustatory responsiveness in *Bombus*. Whether this increase in gustatory responsiveness is reflective of a lowered sucrose response threshold, an increase in motivation to forage, an increase in thirst, or a combination of these traits, cannot be determined from the current protocol. In addition, harnessed bees will accept different concentrations of sucrose compared with free-moving bees (Mommaerts et al. 2013; Mujagic and Erber 2009), and as such, determination of how octopamine affects sucrose acceptability in an ecologically realistic context will need to be addressed in a free-moving protocol in future work. As in *Apis*, effects were dose-dependent: bees fed a higher dose of 10 μl of 8 $\mu\text{g}/\mu\text{l}$ (80 μg) were more responsive to sucrose across nearly all concentrations and initially more responsive to water. Individuals in our lower-dose treatment (10 μl of 2 $\mu\text{g}/\mu\text{l}$ = 20 μg) were not more responsive to sucrose or water than control bees. Scheiner et al. (2002) assayed honey bees using a similar method and found analogous dose dependency. In contrast to our findings with *Bombus*, in this previous work, honey bees showed an increased sucrose responsiveness following exposure to much lower doses of OA (1.9 and 9 μg). In a second study of OA's effects on honey bees, increased sucrose responsiveness occurred following doses of 0.2, 2.0, and 20 μg (Pankiw and Page 2003). In stingless bees, Mc Cabe et al. (2017) compared the sucrose responsiveness of bees following doses of 9.5, 19, and 38 μg OA and reported effects at the lowest doses as well. These differences in effectiveness of the lowest doses are unlikely to be due to differences in protocol, since in all these studies bees were immobilized and responsiveness was measured in a similar fashion. Without further data, we cannot identify the source of this discrepancy. Body size is certainly a plausible explanation, but more subtle differences, for example, differences in receptor type or density, cannot be ruled out. As Mc Cabe et al. (2017) noted, when OA is consumed by honey bees, its behavioral effects are clear, but their etiology is not: OA might change brain titers directly or via more complex

signaling cascades (as Scheiner et al. 2017 showed for TA); likewise effects following consumption leave the role of OA's metabolites open (although work by Barron et al. (2007b) showed that radiolabelled OA consumed by honey bees rapidly makes its way to the brain). In addition to the dose difference noted here, discrepancies between *A. mellifera* and stingless bees in the timing of OA-enhanced sucrose responsiveness were noted by Mc Cabe et al (2017) raising the prospect that OA may exert its effects on gustatory responsiveness differently across taxa.

Also in keeping with previous findings from honey bees, we found that when we used the higher dose of OA (80 µg) in Experiment 2, pre-consumption of OA appeared to enhance learning performance, at least until around trial 8. However, mid-way through the training trials, all bees appeared to have a reduction in motivation, meaning that performance was equivalent in the final, test trial. Previous work on bumble bees has tested individuals over 8 trials (Muth et al. 2018; Riveros et al. 2020); here we attempted 11 trials in order to give bees a greater opportunity to learn; however, bees' motivation across all treatments dropped after the 6th trial. While the PER protocol carries the advantage of being able to tightly control stimulus and reward presentation, it is limited in that the only behavior that is recorded is the bees' tendency to extend its proboscis, which can be confounded with factors aside from learning and memory such as motivation (rev. Muth et al. 2017). As such, the PER protocol is less useful when motivation is likely affecting performance, and in these cases, free-flying assays may be more appropriate (as discussed in Muth and Leonard 2019). Although we attempted to control for motivational effects by removing bees that did not respond to sucrose before starting the learning trials and by excluding bees that did not respond to sucrose more than 4 times across the 11 trials, there were still clear differences in motivation between the two groups (Fig. 4b). Namely, over the course of all trials, OA-fed bees were more likely to extend their proboscis to consume the sucrose reward than control bees (i.e., they showed a differential response to the unconditioned stimulus). As such, the differences

seen between the treatments in bees' tendency to extend their proboscis towards the conditioned stimulus may reflect motivational differences as much as differences in learning aptitude. In addition, bees were less responsive in general in the current learning protocol than has been found in previous work using similar protocols (e.g., (Muth et al. 2018; Riveros and Gronenberg 2012). Work addressing OA effects on learning in a free-moving protocol (e.g., Muth et al. 2017) may combat some of these limitations.

Work from honey bees also suggested that OA may have had the capacity to affect sensory responsiveness to features of both the unconditioned stimulus (US+) and conditioned stimulus (CS+) in ways that could promote learning performance. For example, given that Exp.1 established clear effects on gustatory responsiveness, bees in the treated group might have perceived the value of the US+ as higher value than control bees, a feature that can boost learning performance. It is also possible that OA's ability to increase visual responsiveness (Scheiner et al. 2014) rendered the CS+ more salient to OA-dosed subjects in some way. Further work would be required to pinpoint the driver/s of the apparent performance difference we detected. Going forward, the effects of OA on learning and memory in bumble bees may be better addressed in protocols where bees are free-moving and where motivation vs. learning can be more easily differentiated (e.g., as in Muth and Leonard 2019). While data collected similarly on this apparatus did not detect changes in responses through 8 training trials (Riveros et al. 2020), clearly our bees' participation dropped markedly after the 6th trial, due to satiation, fatigue, or other unknown factors. This led to few responses to the conditioned stimulus in the test phase across both groups, making them difficult to compare and likely obscuring any potential differences.

5. CONCLUSION

Following OA consumption, results found in *Bombus* mirror those reported in *Apis* and *Meliponinae* in relation to sucrose responsiveness

(both genera) and learning performance generally (which has only been measured in *Apis*). Yet, we did note some differences—namely, *Bombus* workers were not affected by our lower dose of OA, which work on the two other genera would have predicted to increase sucrose responsiveness. While subtle differences in OA-mediated behavior may not be significant for understanding broad patterns of aminergic-mediated social organization, we believe they are worth noting for two reasons. First, small changes in appetitive signaling pathways could be meaningful for understanding mechanisms involved in ecological radiation (Ji et al. 2020; Pankiw 2003) as OA is clearly involved in determining what bees choose to collect and their motivation to do so. Secondly, many popular pesticides target OA receptors (Ahmed and Vogel 2020; Farooqui 2013; Papaefthimiou et al. 2013) and the OA signaling pathway in particular has been implicated in mediating bees' responses to stress (Chen et al. 2008; Corby-Harris et al. 2020), pathogens and parasites (Mayack et al. 2015; Spivak et al. 2003), and pollutants (Søvik et al. 2015). In an era of wild bee declines, understanding whether *A. mellifera* is indeed a representative model for anthropogenic influence on aminergic pathways more broadly is a pressing challenge.

SUPPLEMENTARY INFORMATION

The online version contains supplementary material available at <https://doi.org/10.1007/s13592-023-00992-3>.

AUTHOR CONTRIBUTION

ASL conceived of the experiments; experimental design was planned with input from EB and FM. EB collected the data. FM analyzed the data and co-wrote the manuscript with ASL. All authors read and approved the final manuscript.

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DATA AVAILABILITY

Data are uploaded as Supplementary Material.

CODE AVAILABILITY

This study did not generate custom code.

DECLARATIONS

Ethics approval While no ethical approval was needed, we aimed to minimize potential suffering to bees through cold-immobilizing them prior to placing them in harnesses for the experimental protocol. Bees were euthanized via freezing.

Consent to participate Not applicable.

Consent for publication Not applicable.

Competing interests The authors declare no competing interests.

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