

University of Nevada, Reno

The Effects of Octopamine on Bumblebee Responsiveness, Learning, and Memory

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by

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Abstract

Octopamine (OA), the insect ortholog of the human neurotransmitter/ neurohormone norepinephrine, has been shown to regulate motivation, learning and memory in invertebrates. The effect of octopamine on responsiveness has been widely studied in honeybees; however, the effect of octopamine on bumblebee responsiveness is not well established. The ability of a bee not only to sense diminishing levels of floral reward (e.g. sucrose, found in floral nectar), but also to remember the location of such a reward would be beneficial for the bee as well as for a plant which relies on bees to spread its pollen. I propose to test the ability of octopamine to reduce sucrose response thresholds as well as to enhance learning and memory in bumblebees. Exploring this neuronally-mediated process and future searches to detect the presence of octopamine in plant nectar will lead to a better understanding of various chemoattractants used by plants in nature to attract and manipulate the behavior of pollinators.

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Introduction

Learning is a vital skill that influences the survival of many animals (Carew, 2000; Chittka & Thomson, 2001). In a broad sense, learning can be defined as a change in state (i.e. behavior) as a result of experience, and if that change in state occurs due to experiences from the past, we define that as memory (Shettleworth, 2010). Learning, memory, and other processes such as perception and decision-making are all mechanisms by which animals obtain, process, store, and act on information from the environment; collectively these mechanisms are known as cognition (Shettleworth, 2010). The ability to perceive the surrounding environment and adjust behavior as a result of experience is crucial for an animal's success. Classic rewards that reinforce learning, such as food, water, and sexual activity, are all necessary for survival and reproductive success (Bouton, 2007). An animal's fitness can be largely determined by its ability to learn about the surrounding environment and adapt behavior after experience with rewarding and unrewarding stimuli.

Classical conditioning is one form of learning, involving learning to associate a stimulus, such as sound or color, with a biologically significant stimulus, such as food or a shock (Bouton, 2007). A biologically significant stimulus elicits a response that is unconditioned, in other words, an organism will naturally respond a certain way to the stimulus without the need for an association to be made. In Ivan Pavlov's renowned learning experiments, the learned association of food with a ringing bell caused several biological processes within the dog's brain and digestive system to prepare for a meal

resulting in the conditioned drooling response (Bouton, 2007; Carew, 2000; Pavlov, Gantt, & Fol'bert, 1928). Pavlov's experiments highlight how the process of learning depends on both internal and external factors as both biological processing and environmental factors (stimuli) were necessary for the dogs to learn the association. Knowledge of such factors is critical to understanding the mechanisms of learning and memory formation.

An animal's ability to succeed in its environment through behavioral flexibility results from learning associations about environmental stimuli. One such organism where learning is of vital importance to survival is the bee. Bees¹ cope with unpredictable and ever-changing environmental conditions that make learning where to find high quality nectar, their main source of carbohydrates, and high quality pollen, their main source of protein, important for survival. Bees often travel several miles from their colonies to forage, and significant amounts of fuel are required to sustain flight (Carew, 2000; Goulson, 2010). Remembering the location of flowers with high quality nectar is beneficial to a foraging bee needing to fuel up on her journeys to and from the colony. Nectar and pollen availability can change over the course of a season or even over the course of a day; therefore, learning about the rewards of many different types of flowers and knowing when to switch to new flowers at the most profitable time is advantageous for a foraging bee (Leonard, Dornhaus, Papaj, 2011).

In fact, bees – both honeybees (*Apis mellifera*) and bumblebees (*Bombus impatiens*) – have been used as a model system to study cognition since the Nobel Prize

¹ Throughout this thesis, the term 'bee' is used in a general sense to refer to the two main model species of bees used to study cognition, bumblebees (*Bombus impatiens*) and honeybees (*Apis mellifera*). The terms 'honeybee' and 'bumblebee' will be used to discuss the two species individually.

winning work of Karl von Frisch in the early twentieth century (Carew, 2000; Frisch, 1971). Observations that foraging bees, often despite social cues of other foragers, choose the same type of flower to visit when foraging, led to the notion that bees could distinguish between flowers as well as remember the location of reward producing flowers. This observation led researchers to search for the sensory cues that bees use to learn about flowers (Bouton, 2007; Chittka & Thomson, 2001). Using classical conditioning techniques, Karl von Frisch repeatedly paired a honey reward with a blue-colored dish for honeybees to feed from. Following repeated exposure to the honey-filled blue dish, honeybees were placed in an arena with several empty dishes of different colors. The majority of honeybees flew to the blue dish that had been associated with the reward, demonstrating their ability to see and learn about color (Frisch, 1971; Carew, 2000).

Following these discoveries made over a century ago regarding bees' multisensory abilities, bees have emerged as an important model animal to study learning and memory formation. Having established bees' ability to distinguish between floral color cues, researchers conducted experiments to look at the ability for bees to recognize different levels of complexity in floral pattern (Carew, 2000; Giurfa, 1997; Leonard, Dornhaus & Papaj, 2011). Through experiments using classical conditioning to train honeybees to recognize flowers of different colors, shapes, and boundaries, James Gould discovered that honeybees could distinguish between the shapes and spatial elements of the different flowers (Carew, 2000; Gould, 1986). The sophistication of bees' multisensory abilities was further established with the discovery that honeybees could discern between symmetrical and asymmetrical visual patterns— an ability that is limited

to species such as humans, apes, dolphins, and some birds (Giurfa, 1997). The notion that bees make a “mental snapshot” of a whole floral pattern has led to hypotheses that more complex flowers—with distinct color, pattern, and shape—may be more memorable for pollinators and help to ensure pollinator return (Leonard et al., 2011). Modern hypotheses also question how multiple senses, such as visual and olfaction, function together in bee perception and cognition (Leonard & Masek, 2014).

Contemporary bee research utilizes several different methods to study bee cognition in a laboratory setting. Free-moving assays allow bees to explore a restricted environment that mimics their natural environment with flower-type structures. In free-moving learning experiments, bees can be trained to associate aspects of flowers with floral rewards (Muth, Francis, & Leonard, 2016). By imitating a bee’s natural environment, free-moving assays can provide insight to how a bee would normally behave while still providing a relatively controlled setting. Studying bee learning and memory often requires precise stimulus presentation, and a method that offers such a level of control is known as the proboscis extension response (PER) protocol (Giurfa, 2007; Muth, Scampini, & Leonard, 2015). When presented with a sucrose solution via antennal stimulation, a bumblebee extends its proboscis to receive this reward. This response is unconditioned, as a bee naturally drinks from the solution when the reward is presented. If the unconditioned reward is presented while (or shortly before) the bee is exposed to a stimulus, then over time the bee learns to associate the stimulus with the reward. This learned (conditioned) response can be observed as the bee extending her proboscis to the stimulus alone (Giurfa, 2007). The proboscis extension response is a reliable behavioral response that can be observed in a controlled manner by restraining

bees in harnesses while still allowing free movement of their antennae and mouthparts (Carew, 2000; Giurfa, 2007; Hammer, 1997). Through conditioning of the proboscis extension response and the use of the PER protocol, Randolph Menzel and Jochen Erber discovered that honeybees form associations between an odor and a sucrose reward. Associative learning was even observed in the honeybees after a single pairing of the olfactory stimulus and sucrose reward (Menzel & Erber, 1978). Despite not being as reflective of a bee's natural conditions, the proboscis extension response protocol allows for controlled stimuli presentation that can be beneficial to study a bee's associative learning abilities.

To study the ability of bees to alter their behavior as a result of experience, the underlying neural mechanisms of learning and memory formation must be understood. The modulation of behavior in both vertebrate and invertebrates begins in the nervous system with the regulation of sensory and nerve cells (Farooqui, 2012; Roeder, 1999). The effects at one synaptic connection in the brain can change the properties of a whole neural network and result in behavioral modifications (Erber, Kloppenburg, & Scheidler, 1993). The process of synaptic plasticity leading to behavioral and physiological changes begins with specific chemical messengers that facilitate neurotransmission across a synapse. One such chemical messenger that influences multiple biological functions in invertebrate species is octopamine (Farooqui, 2012; Roeder, 1999). Homologous to the noradrenergic system of vertebrates, the octopaminergic system of insects influences multiple physiological events such as the sensitization of sensory inputs, the regulation of motivation, as well as learning and memory formation, all of which are vital to insect survival (Barron, Schulz, & Robinson, 2002; Farooqui, 2012; Pacheco & Breed, 2008).

The natural effects of octopamine on insect behavior and physiology have been widely studied due to the molecule's varied distribution in the insect nervous system as a neurotransmitter, neuromodulator, and neurohormone (Roeder, 1999). The release of octopamine from pre-synaptic neurons influences neural networks by binding to G protein-coupled receptors on post-synaptic neurons. Octopamine binding results in the activation of G proteins and additional secondary messengers that ultimately cause changes in insect behavior (Farooqui, 2012; Roeder, 1999). Octopamine acts as a neuromodulator in the peripheral nervous system by modulating synapses between neurons and muscle fibers, known as neuromuscular junctions. Neuromodulators influence neuron function by potentiating or inhibiting neural signals, but unlike neurotransmitters, they are not directly transmitting the signal. By modulating neuromuscular junctions, octopamine affects muscle contraction, metabolism, and sensory sensitivity (Farooqui, 2012; Evans & O'Shea, 1977). Octopamine was found to enhance excitatory inputs and decrease inhibitory inputs at neuromuscular junctions in locust jumping muscles, thus contributing to strong muscle contractions (Evans & O'Shea, 1977). As a neurohormone, octopamine is released into the hemolymph (insect circulatory system) to mobilize fuel reserves to meet energy demands of flight or other periods of increased activity (Farooqui, 2012; Fields & Woodring, 1991; Roeder, 1999). In one example of fuel mobilization with crickets, an injection of octopamine into the nervous system resulted in increased levels of lipids and carbohydrates in the circulatory system (Fields & Woodring, 1991). In the central nervous system, octopamine's roles as a neurotransmitter and neuromodulator are essential to regulate motivation, arousal,

sensitization of sensory inputs, social behavior, hygiene behavior, and rhythmic behaviors (Farooqui, 2012; Roeder, 1999).

Octopamine plays a role in learning and memory mechanisms in bees, particularly those involved with the proboscis extension response (Hammer, 1993). Within the motor centers of a bee's central nervous system, the VUMmx1 (ventral unpaired median neuron 1) neuron becomes activated when sucrose is presented to a bee's antennae. The subsequent stimulation of nervous system motor centers contributes to the proboscis extension response. Octopamine is the neurotransmitter used by VUMmx1 to transmit the reward signal to the proboscis extension motor centers, effectively telling the bee to stick out its tongue and consume the sucrose reward. In associative learning studies, Hammer discovered that both direct stimulation of VUMmx1 and localized injection of octopamine in the neuron's junction with motor centers had the same effect as a sucrose reward would have: the proboscis extension response was enhanced (Hammer, 1993). Similar to how the bees that had learned to associate an odor with a sucrose reward learned to exhibit the proboscis extension response to the odor alone over time, bees that received direct stimulation of VUMmx1 or a localized injection of octopamine learned to make this association as well. This finding suggests that octopamine has a specific and important role in modulating learning processes in the bee brain.

Octopamine has been shown to influence bee behavior and responsiveness in a variety of studies conducted in honeybees. Reflecting the findings of Hammer, a study conducted at the University of Berlin looking at the effects of octopamine on the responsiveness and activation of particular honeybee brain regions found that the neurotransmitter had an enhancing effect on responses to gustatory and olfactory stimuli

within the antennal pathway (Erber, Kloppenburg & Scheidler, 1993; Hammer, 1993). Octopamine concentration within the antennal lobes of honeybees has been associated with behavioral tasks and shown to be high in bees tasked with foraging (Barron, Schulz, & Robinson, 2002). The consumption of octopamine was found to induce the onset of foraging behavior in young honeybees. Foraging behavior is triggered in young honeybees through colony pheromones, and octopamine was found to enhance the responsiveness to such pheromones, thus resulting in the onset of foraging behavior (Barron et al., 2002). A key study of the effects of octopamine on honeybee responsiveness showed that orally-administered octopamine could significantly enhance responsiveness by reducing sucrose response thresholds: dosed bees extended their mouthparts (proboscis) to consume a solution of sucrose in a lower concentration than non-dosed bees would normally accept (Pankiw & Page, 2003). Studies of the effects of octopamine on honeybee cognition have highlighted the neurochemical's multiple roles in the nervous system that ultimately give rise to behavioral modulation.

While the majority of research on perception and learning has been done with the honeybee (*A. mellifera*), bumblebees (*B. impatiens*) are emerging as an important model system to study cognitive functions as well. Bumblebees and honeybees have different behaviors that require different learning abilities. Bumblebee colonies are significantly smaller than those of honeybees, and as a result, bumblebees are able to take on more general, multi-tasking roles in the colony rather than specialize to one task (Goulson, 2010). Unlike honeybees, bumblebees are able to collect and learn about both nectar and pollen simultaneously (Muth, Francis, & Leonard, 2016; Muth, Papaj & Leonard, 2017). In addition, bumblebees begin foraging shortly after emergence which makes interpreting

learning and memory assays that involve foraging tasks less complex than in honeybees, which only start to forage after two weeks of adult life (Goulson, 2010). Not only do the differences between honeybees and bumblebees warrant the need for separate research to study the different species' behaviors, but recent work has demonstrated a number of advanced cognitive abilities in bumblebees. In a popular study, researchers at the University of Queen Mary London discovered that bumblebees could learn to pull on strings to reach a food source. Naïve bumblebees could watch experienced bumblebees complete the string-pulling task and learn how to reach the food source through social cues (Alem et al., 2016). In a study where bumblebees were trained to transport a small ball to receive a reward, researchers found that not only did naïve observer bumblebees learn how to complete the task from trained bumblebees, but they also exhibited cognitive flexibility by solving the task more efficiently. From a selection of small balls at varying distances from the target, observer bumblebees selected to transport the ball closest to the target even if it was of a different color than previously observed (Loukola, Perry, Coscos, Chittka, 2017). A variety of differences in behavioral and cognitive abilities potentially makes the bumblebee a more useful model for understanding the underlying mechanisms of learning (Muth et al., 2016).

To better understand how particular neurochemicals can underlie mechanisms of learning, in this thesis I use the bumblebee as a model animal to look at changes in responsiveness, learning and memory resulting from octopamine treatment. First, I will determine if orally-administered octopamine reduces sucrose response thresholds in bumblebees, as has been found for honeybees (Pankiw & Page, 2003). If octopamine does enhance responsiveness, then such enhanced responsiveness may correlate with

enhanced learning. Increased learning may occur because bees learn associations faster with greater rewards. If sucrose response thresholds are lowered, then the sucrose will be perceived as being a greater reward and bees will both respond to it and learn associations with it better. Therefore, in my second experiment, I will explore if the effect of octopamine on gustatory responsiveness acts as a mechanism by which octopamine enhances nectar-based learning and memory in bumblebees through classical conditioning of the proboscis extension response.

Materials and Methods

Animals

Colonies of *Bombus impatiens* bumblebees were obtained commercially (Koppert Biological Systems, MI, USA) and housed in a laboratory setting. Each colony was contained in a nest box that was connected to a foraging arena via a tube passageway. Bumblebees were able to travel between the nest box and the foraging arena freely. The foraging arena contained multiple artificial feeders constructed from plastic containers with holes cut into the top for a wick to fit through. The wicks absorbed the sucrose solution within the container for bumblebees to feed upon. The artificial feeders offered a 30% sucrose (in preparation for Experiment 1) or 15% sucrose solution (in preparation for Experiment 2). Artificial feeders were replenished with fresh sucrose every three days or sooner if needed. Each colony received approximately five grams of pollen every other day.

Experiment 1: The effect of octopamine on bumblebee responsiveness

Harnessing

I used the proboscis extension response harnessing technique to test bumblebee responsiveness in a controlled setting. This technique has been established in honeybees by Giurfa and in bumblebees by Riveros and Gronenberg (Giurfa, 2007; Riveros & Gronenberg, 2009). I collected a total of thirty-eight foraging bumblebees – approximately five to ten per trial – from lab-housed colonies using an insect aspirator device, a bee-vac. Only female subjects were obtained as male bumblebees do not forage. Bumblebees were aspirated into a small container with mesh siding to allow for air flow. I put the container holding approximately ten bumblebees on ice for fifteen-twenty minutes to induce short-term paralysis. I placed the cold-anesthetized bumblebees individually into small, plastic tube harnesses with a ‘yoke’ to support the head securely while still allowing proboscis extension (Fig. 1). Bumblebees were left to acclimate to the harnesses for three hours at room temperature in a dark room.

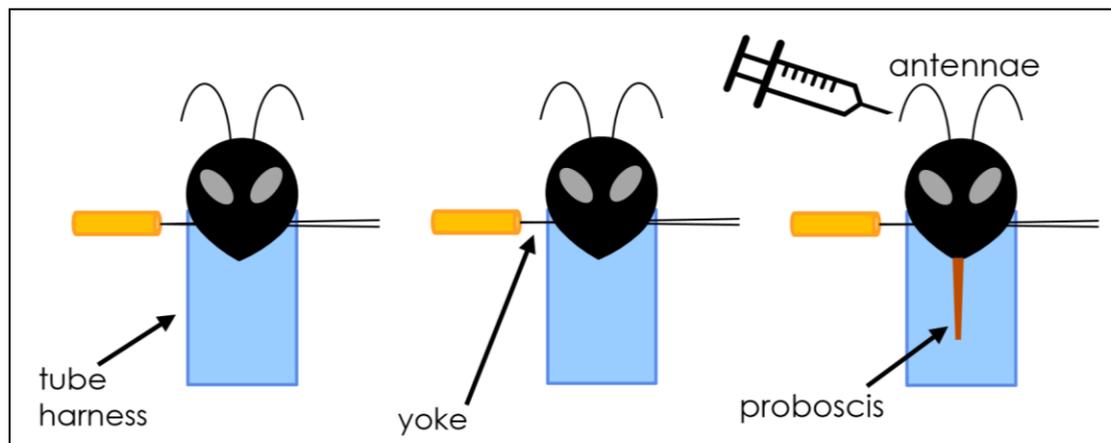


Figure 1. Depiction of harnessed bumblebees for PER assay. Following placement on ice to induce short-term paralysis, bumblebees were placed into tube harnesses. A yoke was placed to offer head support while still allowing for proboscis extension.

Treatments

I used three different treatment groups to determine if octopamine affects sucrose response thresholds in bumblebees. All treatments were fed to bumblebees (via a Hamilton syringe) as oral consumption of octopamine has been shown to have similar effects as direct injection into the nervous system without being as invasive (Barron, Schulz, & Robinson, 2002; Pankiw & Page, 2003). Two of the three groups were fed octopamine but the two octopamine-fed treatments differed by the concentration administered. One group was fed 10 μ l of high levels of octopamine (8 μ g/ μ l) in a 30% sucrose solution (N=13). The second group was fed 10 μ l of low levels of octopamine (2 μ g/ μ l) in a 30% sucrose solution (N=13). A control group was fed 10 μ l of a 30% sucrose solution that contained no octopamine (N=12). Treatments were equally distributed among bumblebees in each trial. Following pre-feeding of the treatment groups, I left the bumblebees to sit for thirty minutes to allow for full absorption of the neurotransmitter as thirty minutes was determined to be the optimal time in honeybees (Pankiw & Page, 2003). Table 1 gives a visual representation of the three treatment groups used in Experiment 1, the three dosages orally-administered to bumblebees of the different treatments, and the number of bumblebees per treatment (Table 1).

Table 1. Representation of the three treatments utilized in gustatory responsiveness testing (Experiment 1).

Treatment	Dosage	Number of Bees
High levels of octopamine	10 μ l of 8 μ g/ μ l OA in a 30% sucrose solution	13
Low levels of octopamine	10 μ l of 2 μ g/ μ l OA in a 30% sucrose solution	13
Control	10 μ l of 30% sucrose solution	12

Protocol

Bumblebee responsiveness was tested in a dark room illuminated only with red light. Bees are relatively insensitive to longer wavelengths, so the red light eliminated any added visual stimuli that may influence responsiveness while allowing the bumblebees' responses to be observed. Following techniques established in honeybees, I measured the gustatory responsiveness of each treatment group by offering bumblebees eight different concentrations of sucrose solution (0.01%, 0.03%, 0.1%, 0.3%, 1%, 3%, 10%, 30%) (Pankiw & Page, 2003). I presented a syringe filled with water to the antennae of each harnessed bumblebee prior to sucrose presentation to account for any effects of octopamine consumption on overall thirst (apart from sensitivity to sucrose). Following water presentation, I presented each bumblebee's antennae with a syringe filled with the varying concentrations of sucrose solution, beginning with the most dilute. Each paired presentation of water followed by sucrose was conducted in five minute intervals (Fig. 2). I recorded bumblebee responsiveness to the different sucrose concentrations by noting any bumblebee that exhibited proboscis extension following the sucrose solution being presented to their antennae. The mean Gustatory Responsiveness Score (GRS) of a given treatment group was calculated by averaging the total number of proboscis (mouthpart) extension responses across the eight concentrations of sucrose being presented to each subject's antennae.

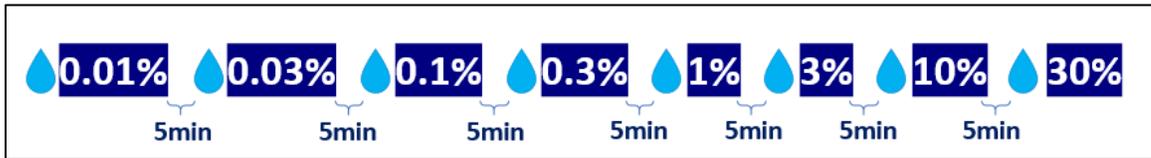


Figure 2. Visual representation of Experiment 1 Protocol. Eight different sucrose concentrations were administered to each bumblebee’s antennae from most dilute to least. Water presentation preceded each sucrose concentration. Concentration presentations were conducted in 5 minute intervals.

Experiment 2: The effect of octopamine on bumblebee associative learning

Harnessing

Prior to placing bumblebees in harnesses, bumblebees were maintained in lab-housed colonies with artificial feeders supplying 15% sucrose. PER assays are normally conducted with 50% sucrose as the reward; however, we used 30% sucrose to see if the responsiveness effects of octopamine were influencing associative learning abilities (Giurfa, 2007). Therefore, we maintained bumblebees on 15% sucrose to make them motivated for 30% sucrose. A total of fifty-five bumblebees were obtained from lab-housed colonies and harnessed as in Experiment 1 (See Fig. 1).

Training Apparatus

To control stimuli presentation for nectar-based associative learning, a training apparatus was used that contained individual testing chambers to house each test subject (Fig. 3). Cylindrical tubing was used to create the chambers, and each chamber contained a small window to allow for reward presentation. Twelve chambers were attached to the

base of a suspended rotating platform. The bottom of each chamber remained open except for a small support system for each harness to be mounted to. A blue LED lighting system was placed below the rotating platform containing the chambers. The light system was constructed to illuminate only one chamber at a time. The chambers were lined with aluminum foil to allow to reflect light throughout the entire chamber. The chambers allowed for precise light presentation to an individual test subject while limiting exposure to the remaining test subjects. The rotating platform allowed for an easy method of moving test subjects into the light field while minimizing movements of subjects within the chambers.

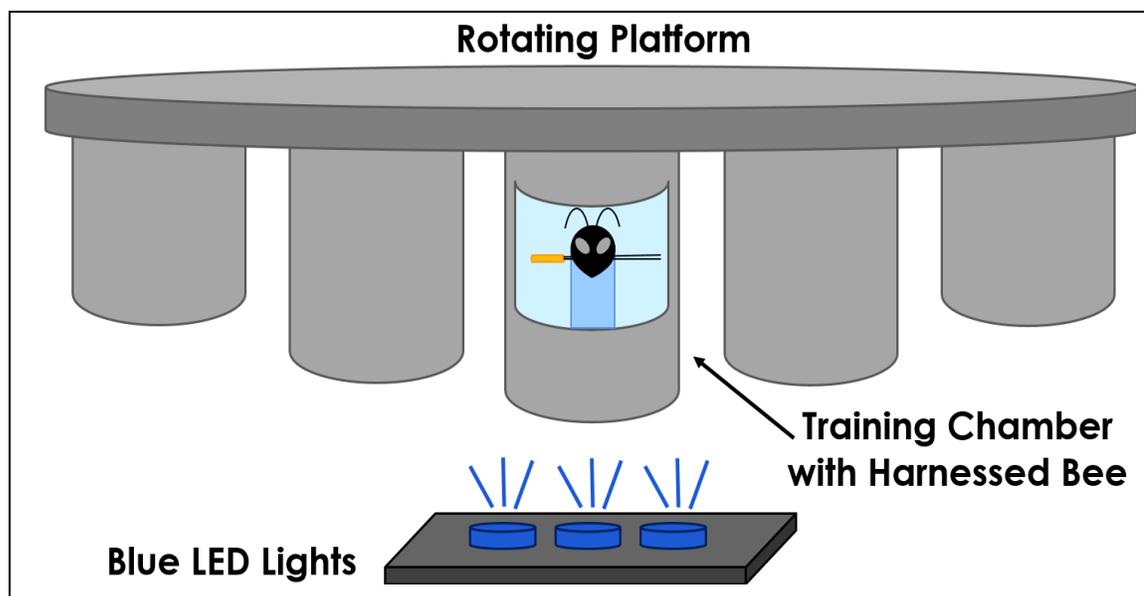


Figure 3. Training apparatus for Experiment 2. Harnessed bumblebees were placed into individual training chambers attached to a rotating platform. Blue LED lights below the training chambers could be manually turned on and off to control visual stimulus presentation.

Treatments

Three different treatments were used to determine if octopamine influences nectar-based associative learning. One group received 10 μ l of a sucrose solution containing octopamine (8 μ g/ μ l) (N=28). Only the high concentration of octopamine (8 μ g/ μ l) in the first experiment was found to enhance responsiveness, so the lower concentration (2 μ g/ μ l) was not used. Two control groups (paired and unpaired) were used. The paired control treatment group was subject to the same reward presentation protocol as the octopamine treatment group. The unpaired control treatment group underwent the reverse protocol, where reward presentation occurred prior to the conditioned stimulus. Both control groups received 10 μ l of a sucrose solution without octopamine (N=27). The high octopamine treatment and the paired control treatment were equally distributed among the bumblebees each training day. Two bumblebees per training day were randomly assigned the control, unpaired treatment. Following pre-feeding, bees were transferred to the training apparatus and left to acclimate for thirty minutes. Table 2 gives a visual representation of the three different treatment groups used in Experiment 2, the dosages orally-administered for each treatment, the reward direction, and the number of bumblebees per treatment (Table 2).

Table 2. Representation of the two treatments utilized in the PER classical conditioning assay (Experiment 2).

Treatment	Dosage	Reward Direction	Number of Bees
High levels of octopamine	10 μ l of 8 μ g/ μ l OA in a 30% sucrose solution	Paired	28
Control (paired)	10 μ l of 30% sucrose solution	Paired	27
Control (unpaired)	10 μ l of 30% sucrose solution	Unpaired	12

Protocol

I used classical conditioning of the proboscis extension response (PER) to compare any differences between octopamine-fed bumblebees and control bees in learning to associate a visual stimulus with a sucrose reward. Each bumblebee was subject to one training phase and eleven trial phases.

Training phase

A naïve bumblebee was presented with the blue LED light for ten seconds without any reward (stimulus period). Any bumblebees that exhibited PER to the stimulus alone, absent of reward, were removed from the experiment. With the stimulus still present, a sucrose reward was presented to the bumblebee for an additional five seconds (associative period). The syringe containing the sucrose reward was first presented to the bumblebee's antenna, and subjects were allowed to drink for up to three seconds in the five second period. The reward and the stimulus were removed at the same time (Fig.4).

Trial phase

After the initial training phase, eleven trial phases were conducted that contained both a testing period and a reinforcement period. Trials were spaced so that test subjects were tested in five minute intervals. Similar to the stimulus period, the testing period involved presentation of the blue light without reward for ten seconds. If PER was elicited during this ten second interval, the reward was immediately presented for up to three seconds of consumption. If no PER occurred in the initial ten seconds, then a

reinforcement period, similar to the associative period, was conducted. The reinforcement period involved presentation of the reward with the stimulus for five seconds with up to three seconds of reward consumption (Fig.4).

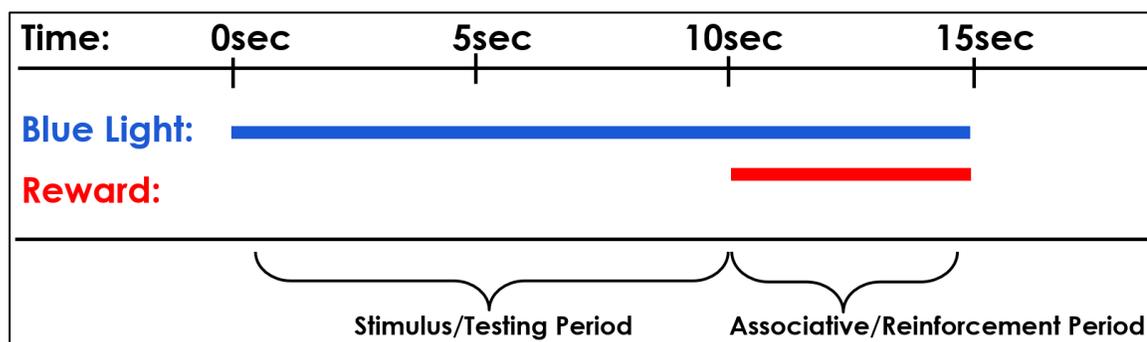


Figure 4. PER Classical Conditioning Protocol. The visual stimulus (blue light) was paired with a sucrose reward. Protocol involved one training phase and eleven trial phases. A training phase included one stimulus period and one associative period. Each trial phase included one testing period and one reinforcement period.

Data Analysis

In Experiment 1, I compared the proportion of bumblebees per treatment group that were responding (i.e. extending their proboscis) to the eight increasing concentrations of sucrose solution. General linear models were used to compare responsiveness at each sucrose concentration and observe any significant differences between treatments. The response variable in this case is the proportion of bumblebees responding, and the explanatory variables are the sucrose concentrations (.01%, .03%, 0.1%, 0.3%, 1%, 3%, 10%, 30%) and treatment ($2\mu\text{g}/\mu\text{l}$ OA in 30% sucrose, $8\mu\text{g}/\mu\text{l}$ OA in 30% sucrose, and no OA in 30% sucrose). Any significant difference between

treatments was further analyzed with Tukey post-hoc tests to determine between which treatments the difference occurred. Chi-square tests were performed to determine differences between the treatments in the percentage of bumblebees responding to water alone. Analysis was performed by R v.3.1.0 (R Development Core Team 2010). In Experiment 2, I compared the percentage of bumblebees exhibiting the proboscis extension response (PER) to the conditioned stimulus (blue light) across all twelve trials. As in Experiment 1, general linear models were used to determine whether bumblebees learned and whether this was affected by whether bumblebees had ingested octopamine or not. The response variable is the percent of PER to the light alone, and explanatory variables are ‘trial’ (1-12) and ‘treatment’ (8 μ g/ μ l OA in 30% sucrose and no OA in 30% sucrose). Chi-square tests were used to compare the frequency of bumblebees exhibiting PER during the reinforcement phase, absent from associative learning, between the two treatments (8 μ g/ μ l OA in 30% sucrose and no OA in 30% sucrose). All analyses were conducted in consultation with Dr. Felicity Muth and Dr. Anne Leonard.

Results

Experiment 1: The effect of octopamine on bumblebee responsiveness

Octopamine-fed bumblebees were more responsive than control bumblebees, but this finding was limited to the bumblebees fed the high-level octopamine treatment (Fig. 5). Overall, bumblebees of all three treatments were more likely to respond to the higher sucrose concentrations than the low sucrose concentrations (GLM: $F_{1, 20} = 25.948$, $p <$

0.0001). However, the proportion of bumblebees from each of the three treatment groups responding to the higher sucrose concentrations differed between the treatments (GLM: $F_{2, 20} = 5.506, p < 0.05$). Bumblebees that were pre-fed a sucrose solution containing high levels of octopamine were more responsive in terms of proboscis extension to varying sucrose concentrations than bumblebees that were pre-fed a sucrose solution without octopamine. This finding is represented by the significant difference in the proportion of bumblebees responding to increasing concentrations of sucrose between the bumblebees pre-fed the $8\mu\text{g}/\mu\text{l}$ octopamine solution and the control, bumblebees pre-fed a plain sucrose solution (Tukey post-hoc test: $p = 0.011$). No significant difference in the proportion of bumblebees responding to the varying sucrose concentrations was found between those pre-fed the low level of octopamine, $2\mu\text{g}/\mu\text{l}$, and the control (Tukey post-hoc test: $p = 0.578$). There is a trend suggesting a difference in the proportion of bumblebees responding to the varying sucrose concentrations between the high and low octopamine pre-fed groups (Tukey post-hoc test: $p = 0.090$). Interestingly, all three treatments showed an increase in the proportion of bumblebees responding to the first sucrose concentration offered (.01%) before dropping down for the following two sucrose concentrations which offer higher rewards (.03% and 0.1%).

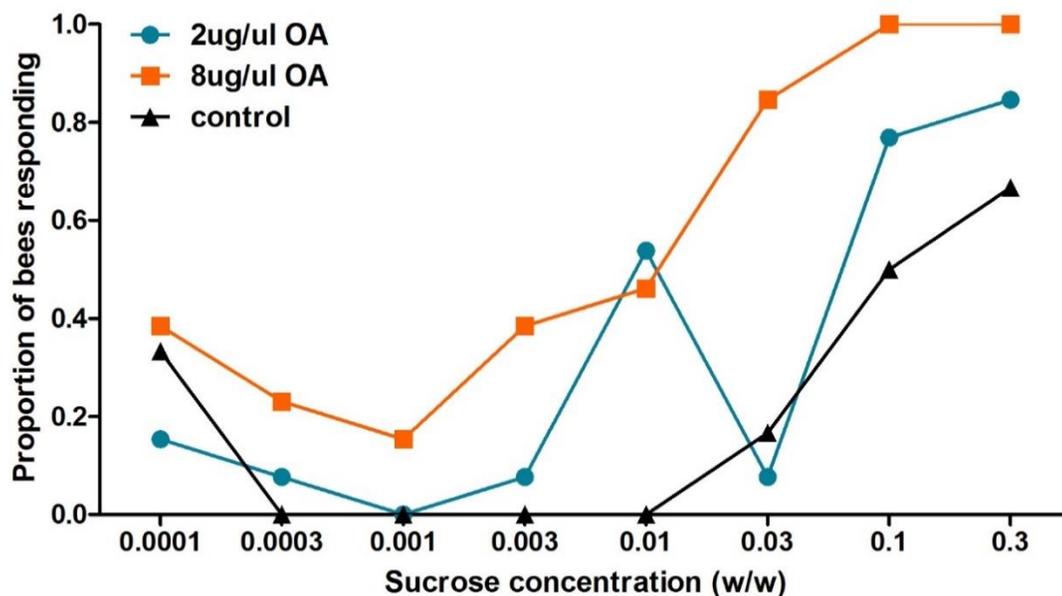


Figure 5. Proportion of bumblebees responding to increasing concentrations of sucrose solution. Response was measured as the proportion of bumblebees exhibiting PER following antennal stimulation of the sucrose concentration. Sucrose concentrations used were: 0.01%, 0.03%, 0.1%, 0.3%, 1%, 3%, 10%, 30%.

Octopamine-fed bumblebees of the high-level octopamine treatment were more responsive to water stimulation in the first trial (prior to the first sucrose concentration presentation) than control bumblebees (Fig. 6). This finding is represented by the significant difference in the frequency of bumblebees from the two treatments (8 $\mu\text{g}/\mu\text{l}$ OA in 30% sucrose and no OA in 30% sucrose) exhibiting PER to the presentation of water, absent of reward, during the first trial ($\chi^2_1 = 6.273$; $p < 0.05$). No significant difference in the proportion of bumblebees responding to water alone was found between the low-level octopamine treatment and the control ($\chi^2_1 = 0.138$; $p = 0.7101$). There is a trend suggesting a difference between the low-level octopamine treatment and the high-level octopamine treatment in proportion of bumblebees responding to water stimulation

($\chi^2_1 = 3.478$; $p = 0.0622$). There is no significant difference in the proportion of bumblebees exhibiting PER to water stimulation in the additional trials (prior to the presentation of the following seven sucrose concentrations).

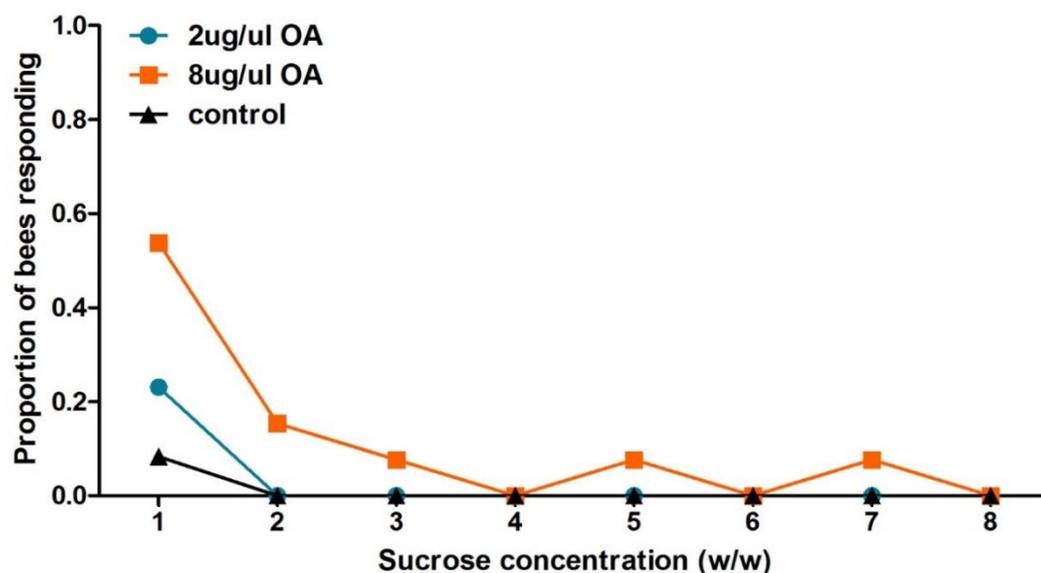


Figure 6. Proportion of bumblebees responding to water stimulation. Response was measured as the proportion of bumblebees exhibiting PER to antennal stimulation with a water-filled syringe. Water was presented to each bee prior to sucrose responsiveness testing with the eight sucrose concentrations, giving eight trials of water presentation (1-8).

Experiment 2: The effect of octopamine on bumblebee associative learning

Octopamine-fed bumblebees learned better than control bumblebees; however, overall learning performance was low for both treatments (Fig. 7). When considering all twelve trials together, there was no significant difference in percent of bumblebees exhibiting PER to the light alone between the successive trials. However, when only considering the first seven trials, there is a significant difference between successive trials (GLM: $F_{1,11} = 20.989$, $p < 0.001$). Significant differences among the different treatments,

the high octopamine concentration and control, were observed in analyses of all twelve trials (GLM: $F_{1,19} = 7.6282$, $p < 0.05$) and the first seven trials (GLM: $F_{1,11} = 16.141$, $p < 0.005$). Overall performance was low for both treatments with less than 50% of bumblebees exhibiting PER to light at any given trial. For both treatments, the percent of bumblebees exhibiting PER to light drops over 20% after trial nine and less than 10% of bumblebees were exhibiting PER to light at trial twelve.

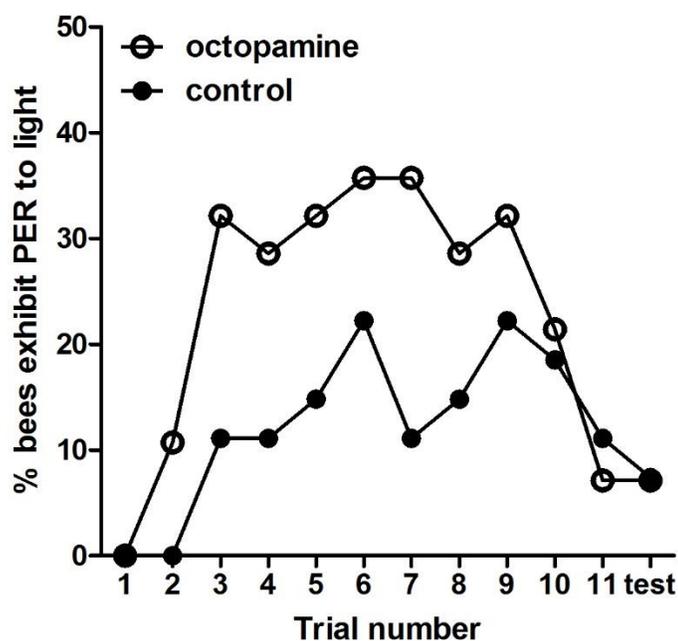


Figure 7. Percentage of bumblebees exhibiting PER to the blue light, absent of reward, per trial. PER was measured during the testing period for each trial (1-12/test) (see Fig. 3).

Because we were concerned with the confounding effect of octopamine on responsiveness as being an influence on learning, we also analyzed the percent of bumblebees responding in each trial separate from associative learning (Fig. 8). By

observing gustatory responsiveness during the reinforcement phase (i.e. bumblebees exhibiting PER during reward presentation), we could confirm results from Experiment 1 and determine any relation between gustatory responsiveness and learning. When looking across all trials and excluding any bumblebees that did not exhibit PER more than two times after being given the sucrose, there is a significant difference between treatments in how responsive bumblebees are, with octopamine-fed bumblebees exhibiting increased gustatory responsiveness compared to the control ($\chi^2_1 = 7.778$; $p < 0.01$). Similarly, a significant difference is observed between treatments in responsiveness when looking at the first eight trials with the octopamine-fed bumblebees exhibiting higher levels of PER to the sucrose reward ($\chi^2_1 = 9.333$; $p < 0.005$).

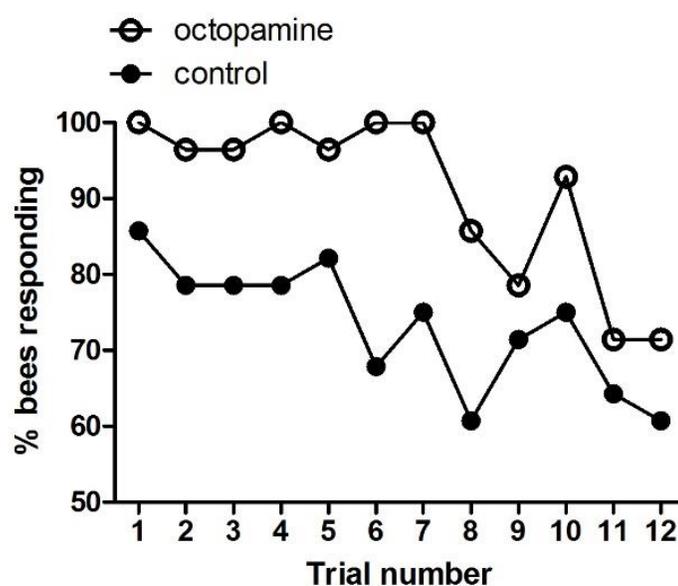


Figure 8. Percentage of bumblebees responding to the sucrose reward per trial. Response was measured as the percent of bumblebees exhibiting PER to the sucrose reward presented during the reinforcement period (see Fig. 3).

Discussion

Octopamine's involvement in associative learning and responsiveness has been established in honeybees; however, until now we have not known what effect octopamine has on bumblebee responsiveness and associative learning in a PER setup. My findings that octopamine increases gustatory responsiveness and improves learning performance highlight the multifunctional role of the neurochemical in the bumblebee nervous system. Research into the mechanisms of bee learning and into the different modulators that increase learning performance is of high importance at a time when bee populations are on the decline.

Experiment 1: The effect of octopamine on bumblebee responsiveness

In Experiment 1, I observed increased gustatory responsiveness in octopamine-fed bumblebees, which mimics the result observed in honeybees (Pankiw & Page, 2003). Bees fed the high concentration octopamine treatment were more responsive to sucrose, across the eight increasing concentrations (.01%, .03%, 0.1%, 0.3%, 1%, 3%, 10%, 30%). Only the bees fed the higher octopamine concentration were observed to have increased responsiveness, which differs from the finding in honeybees which were observed to have increased responsiveness at low doses as well (Pankiw & Page, 2003). There is limited research on how orally-administered octopamine influences bumblebees, as opposed to honeybees, but it is possible that the neurotransmitter needs to be present in higher concentrations to have measurable effects on bumblebees. Interestingly, a similar finding was noticed in one of the few studies looking at octopamine's effects in

bumblebees. In free-flying bumblebees, researchers found that octopamine influenced the length of time it took for bees to learn about a new reward pattern and shift their behavior as a result; however, this result was only evident in bees fed high levels of octopamine (8mg/ml) (Cnaani, Schmidt, & Papaj, 2003). Bumblebees are larger than honeybees, so it is possible bigger doses of octopamine are required in bumblebees to reach the same effects as in honeybees (Goulson, 2010). Further evidence into how orally-administered octopamine is processed and spread to the bee nervous system in comparison to the honeybee nervous system may show variation in the processing mechanisms that cause this difference to be observed.

My finding that bees across all treatments were more responsive to higher sucrose concentrations is expected as the higher concentrations offer a greater level of reward. The initial increase in the proportion of bees responding to the lowest sucrose concentration (.01%) prior to a drop-off in responsiveness seen in response to 0.03% and 0.1% suggests some sort of initial increased responsiveness associated with the pre-feeding. The pre-fed treatments were in 30% sucrose solutions, an increased concentration compared to the 15% that bees were maintained on in their colonies. It is possible that bees remembered receiving this level of a reward during pre-feeding preceded by antenna stimulation, and this experience enhanced later responsiveness to the antenna stimulation with the 0.01% sucrose concentration. The similar initial increase in the proportion of high concentration octopamine-fed bees responding to water alone may be explained similarly. However, the responsiveness to water differs between treatments, so it is possible octopamine increased responsiveness by making the octopamine-fed bees

more responsive to all sensory input, despite the absence of reward (Mercer & Menzel, 1982).

Experiment 2: The effect of octopamine on bumblebee associative learning

In Experiment 2, octopamine-fed bumblebees not only appeared to learn more than control bees, but octopamine-fed bumblebees also appeared to learn more quickly (i.e. after less pairings of the stimulus with reward) than control bees. Despite differences between the two treatments, overall learning performance for bees of both treatments was low (less than 50% at any given trial) compared to other PER learning studies (Giurfa, 2007; Riveros, Gronenberg, 2009). A key difference with our learning experiment was the pre-feeding of the octopamine treatments; it is possible this pre-feeding influenced overall learning performance. In addition, overall learning performance significantly dropped after trial eight. This result is seen in both the percent of bees exhibiting associative learning (responding to the blue light alone) and responding to the sucrose reward during the reinforcement period. This result suggests an overall lack of motivation in the later trials possibly due to the bees becoming more satiated throughout the consecutive trials and less likely to want to consume more sucrose. Despite low responsiveness levels overall, our results still suggest the importance of octopamine in modulating the neural mechanisms involved with learning.

Octopamine's role in associative learning

My findings that octopamine increases responsiveness to sucrose rewards and improves learning performance suggest a relationship between sensitivity to sucrose and

nectar-based associative learning in bumblebees. There are several plausible explanations for the mechanism by which octopamine enhances learning abilities, including both direct and indirect actions of the neurotransmitter. As Hammer showed in his studies of the VUMmx1 neuron, octopamine has a direct role in associative learning by mediating the reinforcement effect during stimulus (reward) presentation, so much so that local injection of octopamine in the honeybee brain has the same reinforcing effects as reward stimulation (Hammer, 1993). As noted earlier, it has been established in honeybees that orally-administered octopamine has similar neuromodulatory effects as local injection in the nervous system (Barron, Schulz, & Robinson, 2002; Pankiw & Page, 2003). Therefore, it is possible that being pre-fed octopamine prior to classical conditioning of the proboscis extension response increases the levels of octopamine in the synapses between VUMmx1 neurons and motor centers of the brain, thus reinforcing the effects of reward presentation and enhancing the proboscis extension response to the light alone.

Octopamine may also have indirect effects on associative learning by enhancing gustatory responsiveness. In both Experiment 1 and Experiment 2, I found that octopamine-dosed bees were more responsive to sucrose, at a variety of concentrations, than control bees. By lowering sucrose response thresholds, octopamine may act as a mechanism to enhance learning by effectively making rewards appear greater to dosed bees and increasing their motivation to respond. Motivation plays a key role in learning. As described by Bouton in *Learning and Behavior*, motivation is the driving force for knowledge to be turned into a behavioral response (Bouton, 2007). For example, a bee may learn that a reward is offered in the presence of the blue LED light; however, the bee might have no reason to exhibit PER until the reward is worth some minimum nutritional

value. Normally, PER assays are conducted with 50% sucrose, as this sucrose concentration provides a greater reward to promote associative learning in bees normally maintained on 30% sucrose (Giurfa, 2007). In Experiment 2, bees were maintained on 15% sucrose prior to experimentation with a 30% sucrose reward. While the 30% sucrose offered a greater reward than the 15% sucrose that bees were accustomed to, 30% sucrose is not nearly as palatable and rewarding to bees as 50% sucrose. Our finding that associative learning was enhanced in octopamine-fed bumblebees despite this lesser reward level suggests that octopamine reduced the bumblebees' sensitivity to sucrose which made the reward appear significantly greater to them, giving them more motivation to exhibit PER which may be driving the result of increased learning.

In addition to enhancing sucrose responsiveness, it is possible that octopamine has another indirect role in learning by enhancing responsiveness to all sensory stimuli. In Experiment 1, I observed an initial increase in bumblebee responsiveness to water stimulation. This observation is significant because water presentation was not paired with a reward, so the result of increased responsiveness likely shows increased sensitivity to sensory input rather than increased motivation for the reward. In a study in honeybees, Mercer and Menzel (1982) found that bees injected with octopamine showed a similar result of enhanced responsiveness. In their study, octopamine-dosed bees showed increased responsiveness to water vapor alone following stimulation of their antenna with sugar water. In this case, the reward was not paired with the water vapor, so the two stimuli were unrelated, yet bees exhibited increased responsiveness indicating sensitization. Mercer and Menzel argue that octopamine acts to increase sensitivity to all sensory input by inducing a generalized excited state in the nervous system. These

researchers also found enhanced responsiveness to an olfactory stimulus after conditioning of a sucrose reward with a particular scent. They argue that enhanced responsiveness is due to the heightened effect of the olfactory stimulus in octopamine-dosed bees. The enhanced responsiveness and increased learning to a visual stimulus in our study may be occurring through the same mechanism of increased sensitivity to all sensory input, whether it be the visual input from the blue light, the gustatory input from the sucrose reward, or the tactile input from antennal stimulation, or a combination of the three. It is possible octopamine induces a state of increased sensitivity causing enhanced responsiveness, thus appearing as enhanced learning.

While the precise mechanisms of how octopamine influences learning, both direct and indirect, may not be entirely known, the resulting behavioral response of orally-administered octopamine in bumblebees is enhancement of learning performance. Octopamine is a naturally occurring neurochemical within the insect nervous system, and by showing how increased levels influence bumblebee learning, I have highlighted octopamine's key role in the neural mechanisms behind the process of learning. As a neuromodulator of learning, octopamine likely has a role in one of the most vital areas of learning for a foraging bee: floral choice. Floral rewards are highly variable; rewards differ not only among plants, but also over different seasons and even different times of the day. The success of a foraging bee depends on her ability to quickly learn associations between prominent floral traits and high quality rewards. These learned associations allow foraging bees to focus their visits on the most profitable flowers; thereby making their foraging bouts more efficient (Leonard, Dornhaus, Papaj, 2011). The speed at which colonies learn floral associations has been shown to directly influence their foraging

abilities, with fast learning colonies collecting significantly more nectar than slow learning colonies (Raine, 2008). Our results suggest high levels of octopamine improve learning speed; therefore, it is possible the mechanism by which certain colonies learned faster was due to higher titers of the neurochemical than slower learning colonies. Further research into learning speed and natural octopamine concentrations would need to be conducted to confirm this hypothesis.

Broader significance of results

By showing that the neuronal mechanism of sucrose responsiveness is similarly mediated in both honeybees (*A. mellifera*) and bumblebees (*B. impatiens*), our findings further shed light on the ability for bumblebees to be an excellent model system to study cognition and behavior particularly in the PER setup. PER assays are primarily conducted with honeybees, but our research contributes to other recent evidence showing that bumblebees can be effectively used as well (Riveros, Gronenberg, 2009). Free-flying assays are beneficial to mimic natural conditions; however, PER assays are beneficial when dealing with neuromodulators that have potential effects on responsiveness to sensory inputs. In such a case, to fully understand behavioral effects, it necessary to control stimulus exposure and minimize additional sensory information as much as possible. Much of the recent work into the advanced cognitive abilities in bumblebees has been conducted with free-flying assays (Alem et al., 2016; Loukola, Perry, Coscos & Chittka, 2017). The PER setup may offer a better understanding of the precise mechanisms and behaviors involved in such advanced cognitive abilities, and our work provides further evidence that such experimentation is possible and informative.

Understanding the cognitive abilities and learning processes in different species of bees is important at a time when bee populations are declining. Two issues facing pollinators are habitat decline and the increased use of pesticides, both of which affect bee learning. As noted earlier, bees learn associations about floral rewards and floral traits which allow them to forage most efficiently in their environment. Habitat loss has limited the abundance of flowers, requiring bees to travel further and more carefully remember the location of high quality rewards. Pesticides, while often beneficial for farming, can have toxic effects on bees. In sub-lethal doses, insecticides have been shown to reduce honeybee and bumblebee learning and foraging abilities (Goulson, Nicholls, Botías, & Rotheray, 2015; Klein, Cabirol, Devaud, Barron, & Lihoreau, 2017). These two forces of pollinator decline highlight the importance of learning for bee sustainability. Further research into different neuromodulators that influence and enhance bee learning would be beneficial in the effort to limit further pollinator decline.

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