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The Olfactory Proboscis Extension Response in the Honey Bee: A Laboratory Exercise in Classical Conditioning

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The beginning neuroscience or psychology student does not often have the opportunity to experiment with classical conditioning. Here I present an inexpensive, easy-to-implement classical conditioning experiment taking advantage of the proboscis extension response to train honey bees to learn an appetitive olfactory association. If an apiary is available, this exercise can be implemented in large scale (training many animals simultaneously) with no specialized equipment so that students can train insects to recognize and respond to a specific odor within the time constraints of a single laboratory classroom session. The proportion of bees that successfully learn the association (40–50%) is considerably lower than in systems utilizing specialized equipment, but the learning is quick and robust enough to clearly demonstrate that learning has occurred. The exercise also lends itself to easy modification to allow alternative learning tasks to be attempted (e.g., multiple odorants, alternative modalities, etc.). Furthermore, this exercise proved to be highly engaging to students.

Key words: classical conditioning; Apis mellifera; proboscis extension response; olfactory learning; classroom exercise; laboratory exercise

In the introductory undergraduate neuroscience classroom, there are generally few opportunities to demonstrate associative learning in animals that result in a response robust enough to benefit the student. It is neither practical nor permissible to bring Pavlov's dogs into the classroom. In the past, we used the California sea hare (*Aplysia californica*) to demonstrate nonassociative learning, specifically habituation involving the gill-withdrawal reflex. This is the system Prof. Eric Kandel made famous in his work leading to the 2000 Nobel Prize in Physiology or Medicine (Kandel, 2004). *Aplysia* are relatively expensive to maintain, however, and we desired demonstrating associative learning rather than nonassociative learning.

There is a long history of both associative and nonassociative learning in invertebrates going back nearly a century. In addition to the honey bee and the sea hare, animals as diverse as the fruit fly *Drosophila melanogaster* (Pitman et al., 2009; Menda et al., 2011), the mosquito *Aedes aegypti* (Menda et al., 2013; Vinauger et al., 2018), the flatworm *Dugesia dorotocephala* (Hovey, 1929; Thompson and McDonnel, 1955), and the earthworm *Lumbricus terrestris* (Ratner and Miller, 1959; Abramson and Buckbee, 1995) have been demonstrated to learn. Invertebrates are often much less expensive to obtain and maintain than vertebrates, and they require little or no regulatory oversight. In fact, invertebrates can be used in a wide range of behavioral studies suitable to the classroom (Abramson, 1986) and yet remain a relatively untapped resource.

Having a research apiary in the department enabled me to develop a classical (Pavlovian) conditioning exercise using the European honey bee (*Apis mellifera*). The proboscis extension response (PER) is a highly robust behavior that is readily elicited within the teaching laboratory setting. Honey bees, like many insects, will extend their proboscis when a sucrose reward is presented to the gustatory receptors of the antennae, mouthparts, or feet. This unconditioned stimulus (US) can easily be paired with a neutral conditioned stimulus (CS) in very few trials (Takeda, 1961; Bitterman et al., 1983; Giurfa and Sandoz, 2012). Within a single laboratory session, students can train several bees to elicit a PER by delivering only the CS, thus demonstrating the learned association. A review of associative learning can be found in Byrne et al. (2014).

Classical conditioning is an associative learning paradigm in which an animal learns to associate two unrelated stimuli. The quintessential example comes from Prof. Ivan Pavlov's work leading to the 1904 Nobel Prize in Physiology or Medicine whereby dogs were trained to associate the sound of a bell (the CS, a neutral stimulus) with the presentation of food (the US, an innately rewarding stimulus) (Pavlov, 1960). We are just barely beginning to understand the learning circuit responsible for this association in mammals (e.g., He et al., 2015). The learning circuitry in insects, however, is considerably more tractable. The mushroom bodies are paired neuropils in insect brains associated with sensory integration, learning, and memory (Erber et al., 1987; Fig. 1A). A simplified depiction of the appetitive olfactory learning circuit within these structures is shown in Fig. 1B. For an animal to learn to associate an olfactory cue (for example) with a gustatory reward, olfactory and gustatory signals must converge spatiotemporally within the brain. Olfactory inputs are initially processed in the primary olfactory neuropils, the antennal lobes. Gustatory inputs are initially processed in the subesophageal ganglion. Projection neurons from these two neuropils synapse onto the dendrites of the Kenyon cells, the intrinsic neurons of the mushroom bodies. Mushroom body output neurons

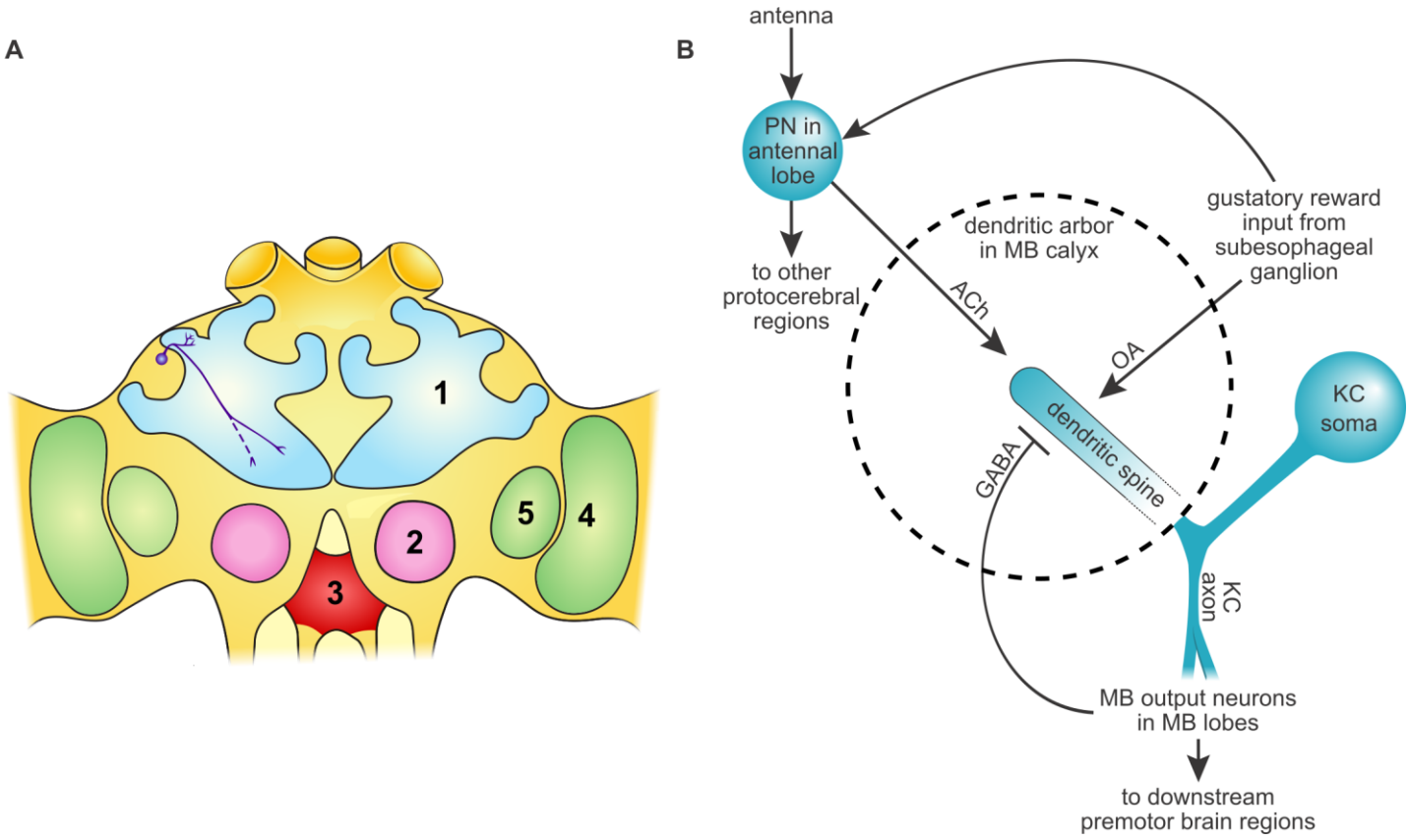

Figure 1. The honey bee brain and the primary appetitive olfactory learning circuit. *(A)* Schematic of the honey bee brain: *(1)* mushroom bodies, neuropils associated with sensory integration, learning, and memory; *(2)* antennal lobes, the primary olfactory processing neuropils; *(3)* subesophageal ganglion, the primary gustatory processing neuropil; and *(4)* the medulla, *(5)* the lobula, and the lamina (between the medulla and the retina, not shown) constitute the optic lobes, the primary visual processing neuropils. One Kenyon cell (KC) is depicted over the left mushroom body. *(B)* The putative appetitive learning circuit. To learn an olfactory/gustatory association, the relevant olfactory and gustatory signals must converge spatiotemporally in the brain. The KCs in the mushroom body likely integrate these signals: cholinergic projection neurons (ACh) from the antennal lobes and octopaminergic projection neurons (OA) from the subesophageal ganglion. Inhibitory γ-aminobutyric acid neurons (GABA) feedback onto the KC inputs. *(A)* modified from Fahrbach and Van Nest, 2016.

transmit information to downstream premotor brain regions to guide behavior. It is thought that modulation of the synapses between the sensory projection neurons and the Kenyon cell dendrites is the mechanism of olfactory appetitive learning in honey bees (Fahrbach, 2006).

In this report, I describe a simple, inexpensive protocol that allows introductory neuroscience or psychology students to train several restrained honey bees to learn to associate an olfactory cue with a sucrose reward in a single two- or three-hour laboratory session. Bees are renowned for their ability to quickly learn an olfactory association in laboratory settings. Bees can form a short-term memory after only a single exposure to a CS, and a life-long memory can be formed after only three exposures (Menzel and Müller, 1996). The simple protocol described here will not result in learning quite at that rate, but learning is unquestionably observed. The students were highly engaged, and the exercise helped solidify not only the concept of classical conditioning, but also the neural circuitry of learning and long-term potentiation. It is often thought that only summer bees—and not winter bees—are able to form these associations so quickly in a laboratory setting. Indeed, reports vary considerably on whether seasonal differences exist in PER-trained honey bees (Ray and Ferneyhough, 1997; Scheiner et al., 2003). This laboratory exercise was performed in late autumn, and the students were asked to consider how seasonal effects might contribute. The results over four years are presented here.

MATERIALS AND METHODS

This exercise was implemented in the introductory neuroscience laboratory class in the fall semesters of 2014, 2015, 2016, and 2017 in the interdisciplinary neuroscience minor program at Wake Forest University (Winston-Salem, North Carolina, USA). There were 23 sections total over four years, averaging 3 sections per semester. There were 2 to 4 groups of students per section, each consisting of 3 or 4 students. Formal assessments were not collected, but informal feedback on student performance and attitudes was recruited from all teaching assistants, and course evaluations were written by the students.

Equipment

Prior to the laboratory exercise, one training kit was assembled for each group of students. Each kit included one scent applicator closed in an empty plastic micropipettor tips box (the "scent box"), one sham applicator left outside the scent box, a wooden applicator stick (Fisherbrand 23-400-112; Thermo Fisher Scientific Inc., Charlotte, NC, USA; toothpicks would work too) sharpened to a point, a square of Styrofoam with a small hole for snugly mounting one harnessed bee during training, and a small vial of 1.5-M sucrose solution.

The scent and sham applicators were clear, 5.8-mL, polyethylene transfer pipettes (VWR 16001-180; VWR International, Radnor, Pennsylvania, USA) with the narrowest, most distal segment removed with scissors. With a micropipettor, 100 µL of peppermint extract (McCormick and Co., Hunt Valley, Maryland, USA) was injected into the scent applicator. The extract was left in the inverted applicator for 15 min and then poured out. Both scent and sham applicators were then washed twice with 500 µL deionized water. A small rubber band was wrapped around the scent applicator, to visually differentiate it from the sham applicator, and the scent applicator was then stored on a sheet of tissue paper in the scent box to minimize escape of the scent. The sham applicator was not stored inside the scent box. Scented and unscented air was delivered by quickly squeezing the bulb of the applicator. Applicators remained effective for several days.

Up to 12 harnesses were prepared for each group, modeled after Dobrin and Fahrbach (2012). Plastic drinking straws approximately 13 mm in diameter were cut to lengths of approximately 75 mm, and a small window was cut at one end approximately 5 mm across and 3 mm deep to allow for the proboscis to extend and to allow access to the bee's mouthparts to deliver sucrose rewards. A pair of holes was pierced into the side of the straw on either side of the window using BioQuip No. 4 insect pins (1208B4; BioQuip Products, Rancho Dominguez, CA, USA). One pin was left in place in the rear holes. See Fig. 2.

Each group had a timer, typically on a cell phone.

Animals

Approximately 6–8 hours before class, worker honey bees (*Apis mellifera*) were captured from a standard box (Langstroth) beehive either at the hive entrance or just under the inside cover. This was done by trapping them individually under open, inverted scintillation vials as they run around the wooden surfaces. As soon as the bees climbed up the sides of the vials, the vials were lifted quickly and capped. There was enough oxygen in the vials to support the bees for at least an hour. No effort was made to control for the age or behavioral role of the bees (e.g., foragers, nurses, etc.; however, doing so might make for interesting experimental questions for the students).

The vials were immediately brought into the laboratory for harnessing and were chilled on ice, a few at a time, just long enough to anesthetize the bees, typically 5–10 mins. After removed from the ice, the bees remained anesthetized for at least a few minutes and were not fully awake for another 5–10 mins. Each anesthetized bee was removed from its vial and inserted head first into the bottom of the straw. With the straw held inverted, the bee would fall until its head was exposed out of the top. If necessary, the head was very gently grasped with fingers or featherweight forceps (BioQuip 4748; BioQuip Products, Rancho Dominguez, CA, USA) and positioned to ensure its face was visible in the window with the first pin already present immediately behind the head. A second pin was then inserted immediately in front of the bee. The two pins acted as a yoke in front of and behind the "neck" of the bee to restrain it in place, with the bee facing the window (Fig. 2). With practice, 20–30 bees can be harnessed per hour per person. The bees were fed small amounts of 1.5-M sucrose with a sharpened wooden applicator stick and left to rest in a warm, dark, quiet place until class (a 33°C environmental chamber is ideal). It was imperative that the bees were starved at least 6 hours before class so that they were motivated to perform for the sucrose reward. However, if it was necessary to collect bees much earlier than class (> 10 h), they were fed 1.5-M sucrose to satiety with a transfer pipette before being placed in the dark.

Figure 2. A honey bee performing a PER to receive a sucrose reward. The bee is pinned between two insect pins at the end of a plastic drinking straw. The window cut in the straw allows for the PER and for delivering a sucrose reward via a sharpened wooden applicator stick (pictured).

Training

Each group was given a training kit, a microcentrifuge tube rack with harnessed bees, and a datasheet (either hardcopy or online; see Supplemental Material 1). The students were instructed to keep the scent applicator inside its closed scent box whenever scent was not being delivered. For a bee to be able to distinguish a peppermint-scented puff of air from an unscented puff of air, the room must not be filled with peppermint scent. The students kept all the harnessed bees toward the left side of the bench. The Styrofoam block, sham applicator, sucrose vial, and sharpened wooden applicator stick were kept in the middle of the bench. The scent box with the scent applicator was kept toward the right side of the bench, far from the bees. If olfactometers or fume extraction systems

are available in sufficient numbers, control of the odor in the laboratory will be better managed, and learning may occur at much faster rates.

The laboratory procedure is outlined in Fig. 3. Each bee was first checked for proper position in the harness and then repositioned if necessary without removal from the harness. Occasionally, a bee will have turned 90°. When this occurred, we would carefully slide the bee to one side of the straw without removing the pins in order to provide some space in front of the proboscis. Next, each bee was checked for motivation to perform a PER (Fig. 2). No air puffs were delivered in this step. The applicator stick was wetted lightly with sucrose solution and touched to the bee's antennae. If the bee extended its proboscis, the student rewarded the bee by touching the stick to the tip of the proboscis and allowed the bee to lick sucrose solution for 2–3 secs. If the bee did not perform a PER within 5–10 secs, the bee was set aside and excluded from the experiment.

The students commenced the training portion (Trials #1–10) of the experiment as per Supplemental Material 1. The timer was started. For the CS- trials (e.g., Trial #1), one bee was moved from the tube rack to the Styrofoam block, and one exposure of the CS- was delivered (a single puff of unscented air on the antennae by the sham applicator, taking care not to touch the antennae with the applicator). The bee was not given a reward (US-). The students then recorded whether the bee performed a PER or not in response to the CS-. The bee was returned to the tube rack, and the same procedure was repeated with the next

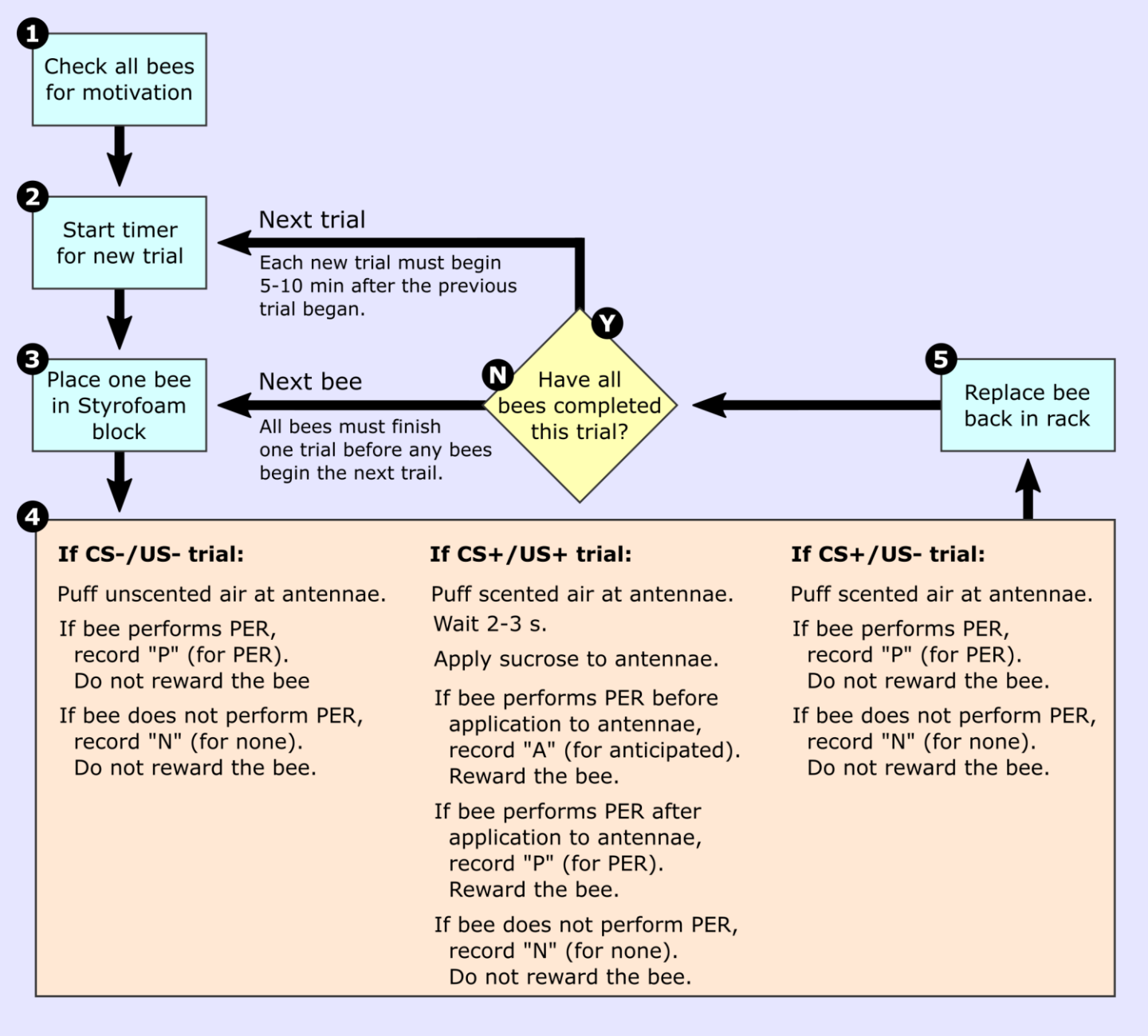

Figure 3. Flowchart of the experimental procedure. Trial conditions are listed in Supplemental Material 1.

bee. All bees in the tube rack completed Trial #1 before Trial #2 was commenced. The interstimulus interval (ISI) was 5–10 mins. If there was extra time at the end of a trial, the timer had to reach 5 mins before commencing the next trial.

The CS+ trials (e.g., Trial #2) required careful coordination of the students. One bee was moved to the Styrofoam block. Then Student A wetted the sharpened stick with sucrose solution. Student B removed the scent applicator from the scent box, delivered a single puff of scented air to the antennae of the bee, and quickly returned the scent applicator to the scent box. Approximately 2–3 secs after scent application, Student A touched the wet stick to the bee's antennae. If the bee performed a PER, Student A touched the wet stick to the bee's proboscis for 2–3 secs. If the bee did not perform a PER within 5–10 s, no reward was given. Student C monitored the bee's behavior continuously through the trial. If the bee anticipated the sucrose presentation and performed a PER prior to the sucrose being applied to the antennae, an "A" ("anticipated") was recorded on the datasheet for that bee for that trial. If the bee performed a PER after sucrose was touched to the antenna, a "P" ("PER") was recorded on the datasheet. If the bee did not perform a PER within the allotted 5–10 secs (and thus no reward was given), an "N" ("none") was recorded on the datasheet. This procedure was repeated for all bees before moving on to the next trial. (It is important for students to understand that a lack of a PER at this point in the experiment was not grounds for removing a bee from the experiment.)

This continued for Trials #1–10 with the US and CS stimuli as indicated in Supplemental Material 1. The extinction portion of the experiment (Trials #11–20) consisted only of trials with the scent applicator (CS+) but no reward (US-) to determine how soon a bee will stop responding with a PER to a stimulus that was once associated with a reward but is no longer rewarded.

Statistics

Generally, the *n*-values for each group were not large enough for meaningful statistical analyses. Thus, students were not asked to perform statistics. Rather, each group was asked to produce two graphs: an apparent rate of learning (proportion of bees performing a PER versus the five CS+/US+ trials) and an apparent rate of memory extinction (proportion of bees performing a PER versus all ten CS+/US- trials). The students were asked to turn in these two graphs as well as a short informal report answering several questions (Supplemental Material 2). Additionally, pooled data from all groups in a class were typically shown to the students (e.g., in the form of Fig. 5B) to better demonstrate learning curves.

For the purposes of this report, statistical analyses were performed on one representative group's data, on the data pooled from all groups from one representative class, and on the data pooled from all groups from all 23 sections over all four years. In each of these analyses, the proportion of bees performing an anticipatory PER in Trial #1 (when still naïve to the CS) was compared separately to the proportion of bees performing an anticipatory PER in every subsequent trial using Fisher's exact test of proportions.

Under an assumption that approximately 4% of naïve bees will perform a PER (Trial #1) and 40% of well-trained bees will perform a PER (e.g., Trial #8), a simple power analysis for a 2-sided comparison of proportions (with $\beta = 0.8$ and $\alpha = 0.05$) would suggest a sample size of $n = 17$ bees should be sufficient to reliably test for differences in proportions at those two stages (Chow et al., 2008).

A Note on Honey Bee Safety

Proper beekeeping PPE should be worn while collecting bees from the hive. In the laboratory, however, it is unlikely to get stung while harnessing the bees. The sting response is controlled in part by an inhibitory circuit; thus, reflexive stinging movements may occur in anesthetized or even decapitated bees. However, if handled carefully, stings are easily avoidable from anesthetized bees. Also, one or two layers of nitrile gloves are known to greatly reduce the ability of a stinger to reach the skin. If this exercise is supervised by experienced bee researchers or beekeepers, risk of getting stung during the preparation phase is minimal.

The likelihood of a student getting stung in the classroom is extraordinarily remote. While the bees are in their harnesses, their stingers are not accessible. Also, only guard bees are aggressive and only at their hive entrance. In all other cases, bees are only aggressive when being attacked. Even then, if a bee can retreat, it will always do so rather than retaliate. In the very unlikely event that a bee should accidently be released from its harness, it will most likely fly toward bright windows or ceiling lights. An instructor may choose to catch it under a cup or beaker, or it may be ignored. It is unlikely to harass the students. We have not had a single escape in class. Nonetheless, because we are a honey bee lab, in the course of our day-to-day field experiments, we are exposed to a large number of bees in various settings, and thus we do have EpiPens available. These are often obtainable ahead of time from campus health services.

RESULTS

Over the four years, 23 groups of students tested a total of 188 bees, averaging 8 bees per group. A representative group's data sheet is illustrated in Fig. 4 with the proportions of bees performing anticipatory PERs plotted in Fig. 5A. Fisher's exact test revealed no differences in the proportion of bees performing an anticipatory PER in Trial #1 versus the proportion in any other trial ($n = 8$; $P > 0.05$ in all cases). This analysis should be taken with a grain of salt, however, as the sample size tested is smaller than a sample size of $n = 17$ suggested by the power analysis. Regardless, a learning trend appears to be evident in the left half of Fig. 5A, and a slight memory extinction trend might be evident in the right half of Fig. 5A.

The results shown in Fig. 5A are from a class with four

TRAINING:			Does PER result (N, P, or A)?							
Trial #	CS	US	Bee #1	Bee #2	Bee #3	Bee #4	Bee #5	Bee #6	Bee #7	Bee #8
1	-	-	N	A	N	N	A	N	N	N
2	+	+	P	P	P	P	P	P	P	P
3	+	+	N	A	N	P	A	P	P	N
4	-	-	N	P	N	N	A	N	N	N
5	+	+	P	A	N	A	N	A	P	P
6	-	-	N	A	N	N	A	N	N	N
7	-	-	N	A	N	N	N	N	N	N
8	+	+	N	A	N	A	N	A	P	N
9	-	-	N	A	N	N	N	N	A	N
10	+	+	P	A	N	A	N	A	A	N

EXTINCTION TESTING:			Does PER result (N or A)?							
Trial #	CS	US	Bee #1	Bee #2	Bee #3	Bee #4	Bee #5	Bee #6	Bee #7	Bee #8
11	+	-	N	A	N	A	A	A	A	N
12	+	-	N	A	N	A	A	A	N	N
13	+	-	A	A	N	A	N	A	N	N
14	+	-	N	N	N	A	N	A	N	N
15	+	-	N	N	N	A	N	A	N	N
16	+	-	N	N	N	A	N	A	N	N
17	+	-	N	N	N	A	N	A	N	N
18	+	-	N	N	N	A	N	A	N	N
19	+	-	N	N	N	A	N	A	N	N
20	+	-	N	N	N	A	N	A	N	N

Figure 4. Sample data for one student group.

groups. Pooling the data from this class resulted in n = 29 bees. The proportions of those bees performing anticipatory PERs on each trial are shown in Fig. 5B. Fisher's exact test revealed no difference in the proportion of bees performing a PER on Trial #1 versus Trial #2 (the first CS+/US+ trial; $P > 0.05$), but there was a difference between Trial #1 versus every other rewarded trial during the training phase (CS+/US+; $P < 0.05$ for Trials #3 and #5; $P < 0.01$ for Trial #8; $P < 0.05$ for Trial #10) as well as on the first two extinction trials (CS+/US-; $P < 0.01$ for Trial #11; $P < 0.05$ for Trial #12). There were no differences between Trial #1 and any of the unrewarded trials during the training phase (CS-/US-; $P > 0.05$ in all cases) or the last eight extinction trials (CS+/US-; $P > 0.05$ in all cases).

Pooling the data from all groups from all four years resulted in n = 188 bees. The proportions of all bees performing anticipatory PERs on each trial are shown in Fig. 5C. Again, Fisher's exact test revealed no difference in the proportion of bees performing an anticipatory PER in Trial #1 versus Trial #2 ($P > 0.05$), but there were strong differences between Trial #1 and every other rewarding trial during the training phase (CS+/US+; $P < 0.001$ in all cases) and on all but the last two extinction trials (CS+/US-; $P < 0.001$ in all cases). There were no differences between the Trial #1 proportions and those in the unrewarded trials during the training phase ($P > 0.05$ in all cases) or in the last two extinction trials ($P > 0.05$ in both cases).

Nearly every student appeared to be highly engaged with the activity. Numerous students mentioned that this was their favorite laboratory exercise in the class. Of 106 course evaluations conducted over the four years, 19 students (18%) indicated the honey bee PER lab was the most valuable. Specific comments include the following:

"The learning in bees stood out to me as the most valuable because it was the first time I was able to classically condition anything. I have heard about it in classes and Pavlov's dog but never got to put the knowledge in to use. Also it was neat to see the difference between summer and winter bees."

"The labs that were the most valuable for me were the earthworm action potentials, cricket vision, and learning in bees. They were most important because they covered concepts and techniques that are relevant to neuroscience research."

"I liked the learning in bees lab, the electrodermal activity, and startle response lab. I think these labs made lecture material more clear."

This lab helped solidify the topic of classical conditioning as well as the concepts of conditioned versus unconditioned stimuli and responses. Informal reports from the students indicated that this activity also paired well with the topics of classical conditioning and long-term potentiation that were presented in the accompanying introductory lecture course (taken as a co- or prerequisite). In fact, the instructors remarked that the students' improved familiarity with learning concepts due to this exercise better equipped them for later discussions in lecture. The students also appeared to gain an appreciation for entomology in general and insect ethology in particular. The general consensus among students was that the bees sticking out their tongues, begging for food, was "cute." Some students even joined our insect neurobiology laboratory and presented independent undergraduate research at a local honey bee conference (e.g., Fraser et al., 2015).

DISCUSSION

The purpose of this report is to illustrate a classical conditioning laboratory exercise that is inexpensive, can be performed in large scale, can be completed by the students within a single classroom period, and clearly demonstrates learning in the animals. Learning did indeed appear to occur; however, the proportion of bees demonstrating a learned association was fairly low (typically between 0.4 and 0.5; Fig. 5). Because winter bees might not learn a classical conditioning task in a laboratory setting as easily as summer bees, one question the students were asked to consider was if autumn bees are different than summer or winter bees (see Supplemental Material 2). There are three likely explanations for these low proportions: (1) autumn bees do in fact exhibit an intermediate phenotype; (2) the population tested was a mixture of summer and winter bees; or (3) the equipment and training protocol described are inferior to other reported experiments. The third explanation is likely true, although not mutually exclusive with either of the first two. There are many reported cases of olfactory appetitive learning in honey bees that result in much better performance (e.g., Smith and Burden, 2014). Scent is poorly controlled in the present protocol. I designed this exercise to be easily and inexpensively performed on several bees simultaneously by several students. Better controlled protocols call for precise olfactometers and carefully designed exhaust systems. If the teaching laboratory has sufficient space at fume hoods, one might try this protocol in the opening to the hoods as a means of more effectively removing scent after exposure. It is also not too difficult to use flexible aluminum ductwork for clothes dryers with small electric fans to move air. The bees can be trained immediately in

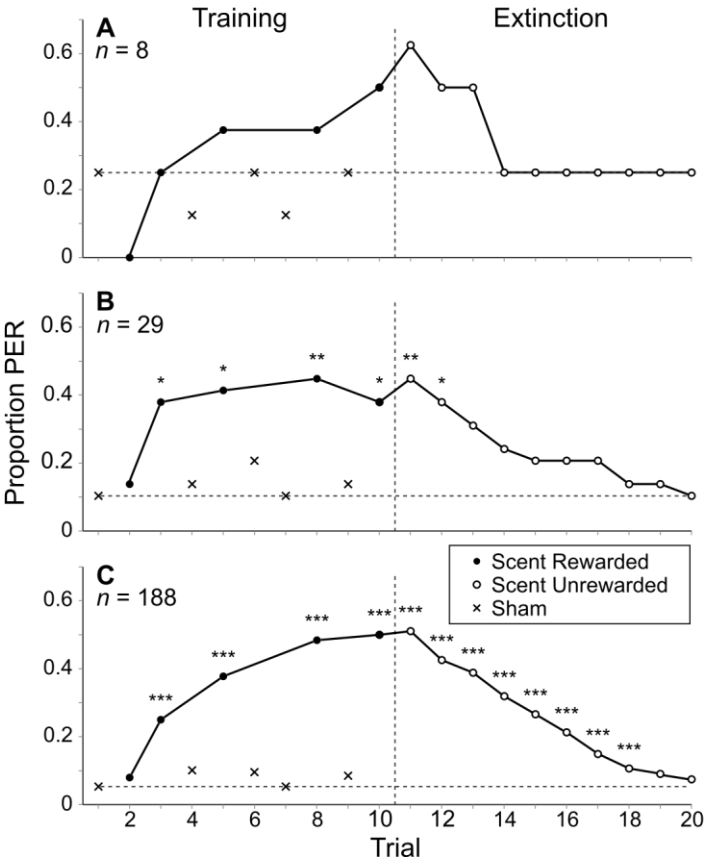

Figure 5. Learning and extinction curves. Trials #1–10 (left of the vertical dashed line) are the training phase of the experiment; Trials #11–20 (right of the vertical dashed line) are the extinction phase. Closed circles represent trials with scented air puffs and a sucrose reward (CS+/US+). Open circles represent trials with scented air puffs without a reward (CS+/US-). X symbols represent trials with unscented air puffs and no reward (CS-/US-). In each panel, for each trial after Trial #1, the proportion of bees making an anticipatory PER was compared (using Fisher's exact test of proportions) to the proportion of bees making an anticipatory PER in Trial #1. The horizontal dashed line represents this Trial #1 proportion. *(A)* The data from Figure 4, one student group. A learning trend and possibly an extinction trend appear to be evident but were not significant. *(B)* Data pooled from 4 student groups in one class section. There was no difference in the first rewarding trial (Trial #2). The proportion in every rewarding trial thereafter was significantly higher as it was in the first two extinction trials. There were no differences in the last 8 extinction trials or any of the sham trials during training. *(C)* Data pooled from all student groups, all class sections, from all four years. There was no difference in the first rewarding trial (Trial #2). There was a significant difference in every rewarding trial thereafter as well as in all but the last two extinction trials. There were no differences in the last 2 extinction trials or any of the sham trials during training. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

front of the open duct, and the duct can exhaust into a nearby fume hood or out a window. Other inexpensive methods for training bees have been previously reported (e.g., Abramson et al., 2007), but resulting data were not generally presented. One final benefit to the present protocol is that it does not use adhesive tape to restrain the bees. After training, the bees can generally be returned to the hive with minimal damage.

It should be mentioned that season is not the only factor affecting a bee's likelihood of learning PER in the laboratory. It is well understood that a large variety of factors—both endogenous and exogenous—affect a bee's sucrose sensitivity and ability or willingness to perform. These include division of labor and behavioral role in the hive, the presence or absence of various hormones and pheromones, interstimulus interval and number of trials, nutritional status of the bee, genotype, and prior experience (see the extensive review by Frost et al., 2012, and the references therein). Honey bees are highly polyandrous. The queen mates with an average of 12 drones and sometimes many more (Tarpy et al., 2004; 2013), and we know that different subfamilies preferentially forage for different resources (Robinson and Page, 1989) at different times of the day (Kraus et al., 2011). Furthermore, each individual has had a unique set of experiences: seeing different things, smelling different things, learning previous associations, etc. Bees are not simple reflex machines. Each acts as a unique agent and will likely make different choices in response to similar stimuli (Van Nest and Moore, 2012). Individual variation, whether genetic or otherwise, should always be kept in mind with ethological studies. It should also be pointed out to the students that one cannot assume a bee did not learn the association simply because it did not perform a PER; the bee might instead lack the motivation or ability to do so.

That honey bees can be trained to associate a specific odor with a food reward in such a short period of time should not be surprising. Insects are capable of a wide variety of complex learning tasks both in the laboratory and in natural settings. Honey bees can learn the location of a food source by following a nestmate's waggle dance (a symbolic communication system) tactilely in a dark hive (von Frisch, 1967). Honey bees can memorize the number of identical feeders to fly past to find their rewarding feeder—i.e., they can count (Chittka and Geiger, 1995). Honey bees can learn to recognize complicated geometric patterns (Greenspan, 2007; Giurfa, 2012), can learn abstract relationships such as same/different or above/below (Giurfa et al., 2001; Avarguès-Weber et al., 2011), and can even learn two such rules simultaneously (Avarguès-Weber et al., 2012). Desert ants (*Cataglyphis fortis*) can count footsteps (Wittlinger et al., 2006). Bumble bees (*Apis terrestris*) can learn a complicated foraging task by watching a nestmate perform the task (Loukola et al., 2017). Rock ants (*Temnothorax albipennis*) actively teach recruits the locations of food sources in a strategy called "tandem running" (Franks and Richardson, 2006). Insect learning is ubiquitous, and complex learning appears to be widespread among the hymenoptera.

The protocol as described herein is simple. There is a multitude of other tests students may explore: effects of different sucrose concentrations as the US; using two different scents as CS+ and CS-; effects of drugs on learning ability (e.g., caffeine, quinine, ethanol); effects of complex scents (e.g., a carefully mixed cocktail of odorants) versus simple scents (e.g., hexanol alone); effects of changing the interstimulus interval, etc. While

using olfaction is probably the easiest and most effective modality in appetitive PER training, other modalities have been successfully employed (e.g., vision: Dobrin and Fahrbach, 2012; thermal sensation: Hammer et al., 2009; tactile sensation: Erber et al., 1998). These would be more challenging to perform, but the ambitious student may wish to try. It would also be valuable to introduce to the students statistical tests of proportions (or comparisons of counts) such as Fisher's exact test of proportions (as performed here) or simple chi-square tests. It should be noted, however, that while Fisher's exact test is generally valid on smaller sample sizes than chi-square tests, it is computationally intensive and relatively unintuitive.

Should honey bees not be available, the instructor may wish to attempt this protocol (adjusting the harness as necessary) with alternative insect species. Classical conditioning utilizing the PER has been demonstrated in a variety of insects including the bumble bee *Bombus impatiens* (Riveros and Gronenberg, 2012; a species commercially available for greenhouse pollination services), the fruit fly *Drosophila melanogaster* (Lofdahl et al., 1992; Pitman et al., 2009), the common housefly *Musca domestica* (Abramson et al., 1996), and several different moth species (Hartlieb, 1996; Fan et al., 1997; Daly and Smith, 2000). Consider, however, that these other species are not known to learn olfactory classical conditioning with the speed and robustness of the honey bee.

Hands-on laboratory exercises are not new in the biological sciences. The lessons learned here in classical conditioning can be taught in any lecture or introductory textbook. However, observing biological phenomena—indeed, manipulating biological phenomena—engages students in ways that passive learning about such phenomena cannot. As noted in the Results section above, we had great success engaging students in an area of biology (insect neuroethology) they likely would never have encountered otherwise, and the students directly facilitated and manipulated animal learning in an intimate, hands-on approach. Active learning improves attitudes toward the subjects being studied (Armbruster et al., 2009). This was clearly evident here. Fostering curiosity and a desire to perform research is critically important in undergraduate science education.

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