ARTICLE
Aversive and Appetitive Learning in *Drosophila* Larvae: A Simple and Powerful Suite of Laboratory Modules for Classroom or Open-ended Research Projects

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A key element of laboratory courses introducing students to neuroscience includes behavioral exercises. Associative learning experiments often conducted in research laboratories are difficult to perform and time consuming. Commonly, these experiments cannot be performed without extensive instrumentation or animal care facilities. Here, we describe three distinct laboratory modules that build on simple chemosensory and memory assays in *Drosophila* larvae. Additionally, we describe open-ended research projects using these assays that can be developed into semester long independent research experiences. Given that *Drosophila* is a genetic model organism, these simple behavioral assays can be used to generate multiple hypothesis driven projects aimed at identifying a gene or class of neurons involved in appetitive and aversive learning. These lab modules are ideally suited for undergraduates at all levels to experience and can be incorporated in a lower/upper level neuroscience course or as a high school outreach exercise. Further, these modules enable students to collect their own data sets, work in groups in collating large data sets, performing statistical comparisons, and presenting results in the form of short research papers or traditional laboratory reports that include a short literature review.

The fruit fly, *Drosophila melanogaster* is a versatile model organism that has been used in biomedical research for over a century (Bellen et al., 2010). Many tools have been developed, refined and implemented that can aid understanding of a broad range of biological processes from the perspective of single genes and cells. In addition to the wide array of tools to study neuroscience, the ease of rearing and manipulating this organism makes it highly suitable for undergraduate laboratories and outreach projects (Berni et al., 2010; Pulver et al., 2011a; Pulver et al., 2011b; Hales et al., 2015; McKellar and Wyttenbach, 2017). Here, we describe three experimental modules using *Drosophila* larvae aimed at elucidating the basic concept of classical conditioning with enough flexibility to design larger hypothesis driven student projects.

The *Drosophila* larva is a simple model organism with features that make it an ideal organism for studying the neurobiology of behavior. The nervous system of a *Drosophila* larva has ten to one hundred times fewer cells than an adult fly, and 10 million times fewer cells than humans (Li et al., 2014). The simplicity of *Drosophila* larvae makes understanding and exploring associative plasticity on a synaptic level a feasible endeavor within an undergraduate classroom setting. The larval nervous system contains approximately 10,000 neurons, about 2,000 of which are found in the brain and rest of them in the ventral cord (Li et al., 2014). Genetic tools to target these neurons and their expression patterns are also available for in-depth studies of the neural basis of behavior (Li et al., 2014; Eichler et al., 2017).

The relative simplicity of the nervous system and the array of genetic tools available make *Drosophila* larvae a powerful and promising model to study neural basis of innate, learned and social behaviors. The larvae at different stages of development have varied feeding behaviors and have provided insights into neural systems underlying feeding preferences and active foraging strategies (Shen, 2012; Kim et al., 2013; Huckesfeld et al., 2015; Kim et al., 2017; Surendran et al., 2017). The larvae have also been a great model to study gustation, thermosensation, nociception and chemosensation (Louis et al., 2008; Bellmann et al., 2010; Oswald et al., 2011; Kim et al., 2013; Grewal et al., 2014; Kim et al., 2017; Yoshino et al., 2017). In addition to sensory behaviors, motor programs such as turning, crawling and forward locomotion have been well-characterized in larvae and can be correlated to neural activity of the CNS in semi-intact preparations (Pulver et al., 2011a; Berni et al., 2012; Huckesfeld et al., 2015; Pulver et al., 2015).

Here we focus on the adapting some of these published behavioral paradigms using the *Drosophila* larvae in study of innate and learned behaviors in the undergraduate classroom (Scherer et al., 2003; Michels et al., 2005; Gerber and Stocker, 2007; Gerber et al., 2009; Chen et al., 2011; Schleyer et al., 2011; El-Keredy et al., 2012; Gerber et al., 2013; Rohwedder et al., 2016; Eichler et al., 2017).

The underlying basis of learning lies in molecular- and circuit-level neuronal changes (Kandel et al., 2014) and these levels of investigation are difficult in rodents and Aplysia behavioral models especially in a traditional teaching laboratory classroom. In this lab, we have adapted the appetitive and aversive associative olfactory learning in *Drosophila* larvae as structured laboratory modules that can be typically performed in 3-4-hour lab classes. Furthermore, we describe genetic strategies to design open ended research projects that can easily be adapted by instructional faculty for a semester long project experience.

One of the most well-studied forms of associative learning, Pavlovian conditioning, requires creating an association between a biologically potent stimulus—that is, a stimulus that causes a behavioral response without learning (e.g., food causes salivation)—and a previously neutral stimulus. When the previously neutral stimulus elicits the response that the biologically potent stimulus usually causes, an association has been established and

learning has occurred (reviewed in (Domjan, 2005).

In the current experiment, we conditioned *Drosophila* larvae to associate reinforcing biologically potent stimuli (sweet and bitter tastants) with neutral odor stimuli, in order to investigate the value of reward and punishment in associative learning in an undergraduate lab setting. Two edible compounds that have been established as reinforcers in associative olfactory learning, fructose (FRU) as the rewarding stimulus and quinine (QU), a bitter compound, as the punishing stimulus, were used in these experiments (Gerber and Stocker, 2007). N-amyl acetate (AM) and 1-octanol (OCT) were used as odors with neutral biological potency at concentrations that were tested to be neither aversive nor appetitive to the larvae as shown previously (Gerber et al., 2013).

We predicted that conditioning would result in larvae developing a preference for odor associated with the reward (appetitive learning) and an aversion to the odor that has been associated with the punishment (aversive learning). In order to correctly interpret the conditioning experiments, we also conducted preliminary locomotor (qualitative) and chemosensory assays (quantitative) to test the potency of the tastants and ability of larvae to navigate and differentiate odors.

Here, we suggest several different experiments using these simple behavioral modules that can also be conducted as part of a semester long research experience to identify and characterize the neural and genetic basis of associative learning in *Drosophila* larvae. These experimental modules were conducted in a teaching lab and a regular classroom with no lab equipment and with a high level of reproducibility and minimal preparation time before experiments. Each of these modules can be completed in 45 mins-3 hours making them ideal for laboratory classes where students can acquire their own data and share analysis and interpretation with their peers. All the data presented in the results was generated by undergraduate seniors between Spring 2015-Spring 2017.

MATERIALS AND METHODS:

Agarose (A9539, Sigma)
Fructose (F0127, Sigma)
Quinine (145904, Aldrich)
n-amyl acetate (AC149182500, Acros Chemicals)
1-octanol (297887, Sigma-Aldrich)
Paraffin oil (18512, Sigma-Aldrich)
Petri Plate (100X15mm) (32-107, Genesee Scientific)
Fly food: Nutri-Fly MF (66-116, Genesee Scientific)
Fly food vials: Narrow (32-116, Genesee Scientific)
Polystyrene weighing dishes (Z186856, Aldrich)
Microwave oven
Glassware
Weighing balance
Pipettes (2-200ul and 200-1000ul) and tips
Gloves
CantonS/CS and Synapsin flies from Bloomington Drosophila Stock Center (CS: Stock id 64349, Syn97CS: Stock id 29031, UAS-TNT: Stock id 288996, 28897, UAS-dTrpA1: Stock id 26263).

Drosophila or “vinegar flies” have a four-stage life cycle; egg, larva pupa, and adult fly (Pulver et al., 2011b; Roote and Prokop, 2013; Hales et al., 2015). Once fertilized, the embryo develops in the egg for around one day (at 25 °C) before hatching as a larva. The larva eats and grows over five days until it pupates and undergoes metamorphosis into the adult fly over the course of four days. Flies can be reared and maintained in culture vials and all stages of the development can be completed in the vial (Hales et al., 2015).

CS wild-type flies from Bloomington Stock Center (Stock # 64349) are reared with media containing yeast, molasses, cornmeal and agar. Culture media is available from Genesee Scientific and small batches can be prepared in the microwave or on a hot plate with a magnetic stirrer (Pulver et al., 2011b). Flies can be maintained in an incubator at 25°C, 60-70% humidity and 12hr/12hr light-dark cycle. If an incubator is not available, flies can be reared on lab benches at room temperature (21°C). This lower temperature can slow down development by a few hours which is largely dependent on stability of the environmental conditions in the laboratory/classroom space (Hales et al., 2015). All the experiments shown here were conducted with flies reared in a 25°C incubator.

Adult flies are allowed to lay eggs for 3 days and transferred to another vial to keep the cultures going. For all the laboratory modules described below we collected 5-day-old larvae (90-120 hours after egg laying) to ensure they remain in feeding stages. *Drosophila melanogaster* larvae used in these experiments undergo essential mid-third instar transition from foraging (feeding) to wandering (non-feeding) behavior at this stage before pupariation and metamorphosis. For reward and punishment learning this transition is critical because wanderers have reduced motivation for feeding and might not perform optimally in feeding related tasks (Ainsley et al., 2008; Gomez-Marin et al., 2010).

Using a spatula, larvae were scooped from the culture vials and dispersed on a weighing dish with distilled water. Using a moist paintbrush, larvae were moved to a second clean weighing dish. This process is repeated until no excess food remains on the larvae. When 50 larvae were collected, they were transferred to a plastic vial containing 5 mL distilled water. Larvae generally show rhythmic movement made up of a series of periodic strides with two phases and can be visualized on 1% agar containing petri plates. In the first phase, the larvae stabilize its center of mass by translocating its head, tail and gut. This is followed by a second phase where body wall muscles show a wave of activity in the direction of movement (Heckscher et al., 2012; Sun and Heckscher, 2016). These movements can be recorded on an iPhone or webcam for visualization to ensure that larvae are healthy and moving comfortably on the agarose substrate before starting the learning experiments (Heckscher et al., 2012; Clark et al., 2016; Sun and Heckscher, 2016). For our experiments we tested larval locomotion on 1% agarose surface but did not characterize the finer aspects of locomotion like velocity, distance travelled, etc. A detailed

description of quantification of these locomotor features can be performed by video analysis as described in (Heckscher et al., 2012; Sun and Heckscher, 2016).

Like vertebrates, adult and larval *Drosophila* fulfill their metabolic and nutritional needs by consuming carbohydrate rich sugars like fructose, glucose, sucrose and other sugars or mixtures available in fruits. In the laboratory, their attraction towards sugars and aversion towards potentially toxic bitter substances can be tested using simple and robust preference assays. Here we describe (module 1) these assays which should be conducted with collected larvae to ensure that sugar and bitter (quinine) substrates used in the assay are sensed and perceived by the larvae as attractive and aversive stimuli.

Module 1: In this module we tested the preference of untrained/naive larvae towards tastants (fructose and quinine) and odorants (Octanol and amyl acetate)
Estimated time: 45 mins- 60 mins

Naïve taste preference of *Drosophila* larvae

For this assay, petri dishes are made with different substrates on either half. Petri dishes are first filled with 1% agarose solution. After the solution has solidified, a knife is used to cut along the midline and remove one half of the agarose gel. The empty half of the dish is then refilled with agarose with 2M fructose or 0.5mM quinine.

These substances have been reported as appetitive or aversive gustatory stimuli, respectively (Apostolopoulou et al., 2015), in *Drosophila* larvae and this simple 2-choice assays enables testing the larvae's gustatory preference**.** Thus, petri dishes should either supply an appetitive and or aversive environment on one half and neutral environment on the remaining half. The dish's midline separates the two environments and can be easily identified visually (Figure 1).

Innate Preference: Using a paintbrush, larvae are moved from the plastic vial and placed on a weigh boat. Ensuring that no excess water is transferred with the larvae, about 30 larvae are placed along the midline of a petri dish. After the lid is closed for five minutes, the number of larvae on either side, as well as the total amount on the dish can be manually recorded.

This is repeated for three trials in assays using fructose and agarose as well as three trials in assays using quinine and agarose as opposing substrates. A preference index is calculated as shown below:

Pref Index (Fructose)= (larvae on 2M fructose - larvae on plain agarose) / total number of larvae

Pref Index (Quinine)= (larvae on 0.5mM quinine - larvae on plain agarose) / total number of larvae

Larvae showing normal movement after collection either show a strong preference for sucrose or an avoidance for quinine (see Fig. 3). This module can be adapted by testing single gene mutations of gustatory receptors involved in sugar and bitter sensation in fruit flies to expose students to genetic basis of chemosensation. Specifically, mutations of GR43a abolish fructose preference in larvae (Miyamoto et al., 2012; Mishra et al., 2013). In addition, to testing sugar attraction and bitter avoidance, the associative learning experiment depends on the ability of the larvae to sense and differentiate odors. To test the naïve odor preference of wild-type larvae a modification of the 2-choice gustatory preference test can be performed as described below.

Module 2: Naïve odor preference of *Drosophila* larvae
Estimated time: 45 mins- 60 mins

The larvae collection and plate set up for these experiments is the same as above except that the entire petri-plate contains 1% agarose. Two pieces of double-sided tape are positioned on opposite sides of the interior surface of a perforated petri lid. Strips of filter paper are placed on either piece of tape. Twenty ul Octanol (Oct; pure or diluted) was pipetted on to one piece of filter paper, while 20ul (pure or diluted) of Amyl acetate (AA) was used on the opposing side (Figure 2). After 30 larvae are transferred from a vial to an agarose-containing petri dish, the lid is closed for five minutes. Since the entire substrate lining the petri dish is agarose in this assay, the odors should present the only variable between opposing sides.

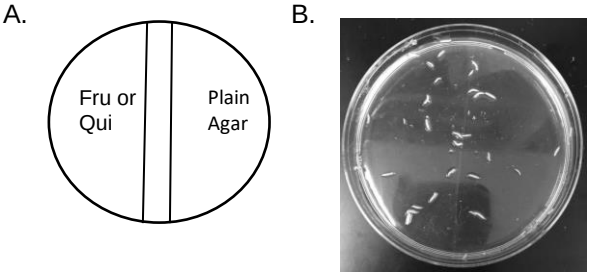

Figure 1. Experimental arena for gustatory choice behavior. *A.* Schematic of petri plate containing 1% agarose with and without fructose (Fru) or quinine (Qui). *B.* Petri plate showing crawling larvae making a choice between fructose (2M in 1% agarose) and plain (1% agarose). Larvae that remain at the midline by the end of trial were not included in the measurements.

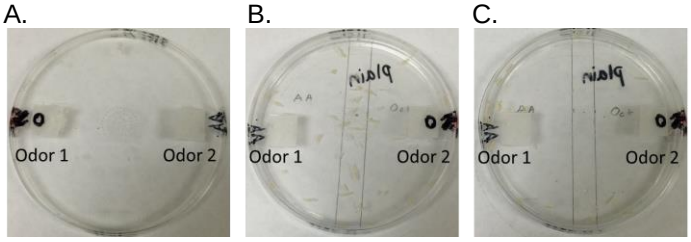

Figure 2. Experimental set-up showing petri-plates with agarose and odorant-soaked filter paper on the lid. Odor 1 was 1:50 (n-amyl acetate) and Odor 2 (Octanol, undiluted). *A.* Experimental arena with agarose and two odors. *B.* Plate showing freshly transferred larvae in the center and *C.* Larvae at the end of the experiment (5 minutes).

Larvae were allowed to choose between odor sides for 5 minutes and counted to calculate odor preference index. In addition to pure odors we also tried four AA and Oct dilution conditions that were as follows: AA and Oct, AA (1:50) and Oct, AA and Oct (1:50), and AA (1:50) and Oct (1:50). Dilutions were made with paraffin oil. Three trials are performed for each of the four dilution conditions and preference index was calculated as described above (See Fig. 4).

This module can be adapted by testing single gene mutations of olfactory receptors involved in sensing a wide variety of odorants in fruit flies to engage students in an open exploratory project aimed at understanding genetic basis of olfaction (Gomez-Marin et al., 2010).

Module 3: Appetitive and Aversive Learning
Estimated time: 90 mins- 120 mins

The associative learning experiment is based on a training phase, where each of the odors is paired with a reward (fructose) or aversive stimuli (quinine) followed by a testing phase where trained larvae are exposed to the odors. The CS, conditioned stimulus (odor) and US, unconditioned stimulus (fructose or quinine) pairing during training follows a spaced training protocol which involves three 5-minute training steps followed by one 5-minute test phase to evaluate memory of odors associated with appetitive (fructose) and aversive (quinine or NaCl) stimuli.

For these experiments larvae were divided into two groups. Fructose was identified as a positive, reward, or appetitive stimulus, so odors associated with fructose was labelled as (+), Quinine being the aversive stimulus, odors associated with quinine was labelled as (-).

Training: Group 1 is trained by presenting AA together with the food reward (fructose: FRU), while OCT is presented without reward (AA+/OCT). Group 2 is trained reciprocally (AA/OCT+). In the subsequent test, it is examined how the larvae of the two groups distribute between AA and OCT in a choice situation.Performance Index were calculated as:

PREF $AA_{AA+/OCT}$ = (animals on AA side - animals on OCT side) / total number of animals (= PREF AA $_{OCT/AA+}$)
PREF $AA_{AA/OCT+}$ = (animals on AA side - animals on OCT side) / total number of animals (= PREF AA $_{OCT+/AA}$)

Performance Index: (PREF $AA_{AA+/OCT}$ + PREF $AA_{AA/OCT+}$)/2

Test: In order to evaluate the effects of reward and punishment, PI scores were averaged across trials for reward or punishment conditions. Thus, PI values were calculated for 2,4,6,8 and 10 minutes after training. Negative PIs associated with aversive learning represent conditioned avoidance. On the other hand, positive PIs associated with appetitive learning represent conditioned approach towards reward associated odor (See Fig. 5).

Independent research projects
To explore the neural basis of above behaviors we can conduct these experiments by manipulating subsets of neurons in the fly brain using the UAS-GAL4 approach (Brand and Perrimon, 1993). This transgenic expression system requires setting up genetic crosses between two fly lines, one expressing the yeast transcription factor GAL4 in specific group cells controlled by a particular enhancer and the other carrying a transgene called upstream activating sequence (UAS) driving the gene of interest. Gal4 drives expression of the transgene (gene of interest downstream of UAS) in the same cells in which Gal4 itself is expressed. Different manipulations (activation, silencing, gene-silencing etc.) of a defined set of cells targeted by a GAL4 line can be made using different UAS-transgenes and identical manipulation can be made in different neuronal subsets by using different GAL4 lines (White and Peabody, 2009). The step-wise methodology of setting up and tracking these crosses have been described in (Roote and Prokop, 2013). A comprehensive list of GAL4 lines targeting different neurons is available for ordering from Bloomington Drosophila Resource center (https://bdsc.indiana.edu/stocks/gal4/index.html). Several of these Gal4 lines target the mushroom body a region implicated in learning and memory formation in Drosophila larvae (Pauls et al., 2010). Gal4 lines specific for this region can be found by selecting mushroom body in the anatomical classifier field of the flylight website: http://flweb.janelia.org/cgi-bin/flew.cgi.

For instructors and students with limited background in working with *Drosophila*, GAL4 lines targeting the dopamine, serotonin, octopamine/tyramine (invertebrate homolog of norepinephrine and epinephrine) can be a good starting point as these systems have been implicated in learning in vertebrates and invertebrates. UAS lines expressing UAS-tetanus toxin (TNT) can be used to inhibit neurotransmitter release to test the effects of blocking dopamine or serotonin release on the above behaviors. TNT specifically cleaves neuronal Synaptobrevin (n-Syb), which is essential for synaptic vesicle release (Sweeney et al., 1995). UAS-tetanus toxin (TNT) flies can be obtained from Bloomington Drosophila Resource center (Stock no. 28996 and 28897).

Alternatively, students can also conduct a mini-screen in class testing single gene-disruptions induced by transposable p-elements. Students and instructors can explore the flybase.org website and identify genes of interest and order insertional alleles of specific genes to test a role of these genes in above behaviors including taste preference, odor preference and conditioning.

To test the amenability of performing these experiments using single gene mutations we tested a synapsin mutant (syn97CS, stock # 29031) shown to reduce appetitive learning (Michels et al., 2005). Synapsin is encoded by a single gene in *Drosophila* and shown to bind vesicles and cytoskeletal actin elements in forming and maintaining reserved pool of vesicles (Akbergenova and Bykhovskaia, 2007).

Three students who continued on the project as part of independent study tested wild type or synapsin mutant flies and were blind to the genotype they were testing. Students performed the taste preference, odor preference

and sugar learning assays to test chemosensation and chemosensory learning in synapsin mutants as compared to wild type flies (see Fig. 6).

RESULTS

Taste preference in *Drosophila* larvae: Gustation is a major chemical sense critical for organismal survival and plays a critical role in finding food, mates and/or sensing hostile environments. The *Drosophila* larvae is an excellent model to study gustatory preference and neural circuits underlying them as the larvae represent a critical feeding stage in the fly's life cycle. Substrates containing sugar are critical to meet the energy needs of the larvae and hence are highly rewarding.

Previous studies have shown that 2M fructose is rewarding to larvae and this preference can be tested by a simple 2 choice assay described above (Apostolopoulou et al., 2015). We find that naïve wild type CS larvae have strong sugar preference and most larvae navigate to 2M fructose as compared to plain agarose in a 5-minute assay indicated by a positive preference index (Figure 3). As a modification of this experiment, students can try different concentrations of fructose or test other sugars (sucrose, glucose or trehalose) in the gustatory preference assay.

Interestingly, the preference for sugar, specifically, fructose is higher if the assay time is increased to 8 minutes (data not shown) and students can try varying the assay time to study the temporal features of this preference assay (Schipanski et al., 2008). Using the same 2 choice assay we also assayed the avoidance of larvae to Quinine, a bitter compound that has previously been shown to aversive (El-Keredy et al., 2012) (Figure 3).

We find that most larvae navigate away from quinine in the 5-minute assay duration indicated by a negative preference index. The avoidance can be observed as early as 3 minutes (data not shown) and a modification of this assay can be used to study the temporal features of this avoidance behavior.

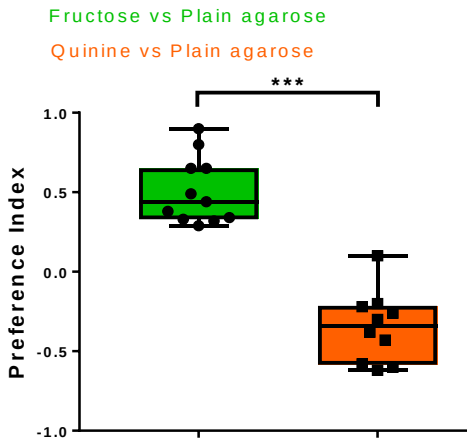

Figure 3. Taste preference in Drosophila larvae. Larvae placed in the center were given a choice between fructose vs agarose and quinine vs agarose. Positive preference indicates attraction to the tastant, while negative preference index indicated aversion. Two groups were compared by a Mann-Whitney U test (n=11, the same two choice assay. We used multiple p<00001).

Odor preference in *Drosophila* larvae:
We next assayed naïve odor preferences of larvae using combinations of concentration of octanol (Oct) and n-amyl acetate (AA) to find concentrations of odors that induce equivalent preference. Previous publications have reported that larvae show equal preference for pure 1- octanol and 1:50 dilution of n-amyl-acetate (Scherer et al., 2003).

We did not find any significant differences between odor avoidances for all concentrations tested except for AA/Oct (1:50) where almost 50% larvae navigated to the AA side (Figure 4). We decided to use AA (1:50)/Oct for learning experiments as the preference for 2 odors was balanced as reported in the literature (Gerber and Stocker, 2007; Gerber et al., 2009; Chen et al., 2011).

Aversive and Appetitive learning:
Once it was ascertained that the larvae collected using the methods described above had normal aversion and attraction to quinine and fructose respectively, we proceeded with the learning assays. The associative performance indices range from −1 to 1, positive values indicate conditioned approach (appetitive learning), and negative values indicate conditioned avoidance (aversive learning).

Figure 5 demonstrates the positive or negative preferences, respectively, towards odors associated with reward or punishment conditioning, respectively.

We tested the memory performance 2,4 ,6,8 and 10 minutes of training and find that the memory performance was strongest at 8 and10 minutes post-training. We found that the memory levels were robust up to 15 minutes of testing but seemed to disappear around 20 minutes post-training (data not shown). As a modification of these experiments, students can try adding more training phases in the spaced protocol to test if the memory can last longer than 10 minutes.

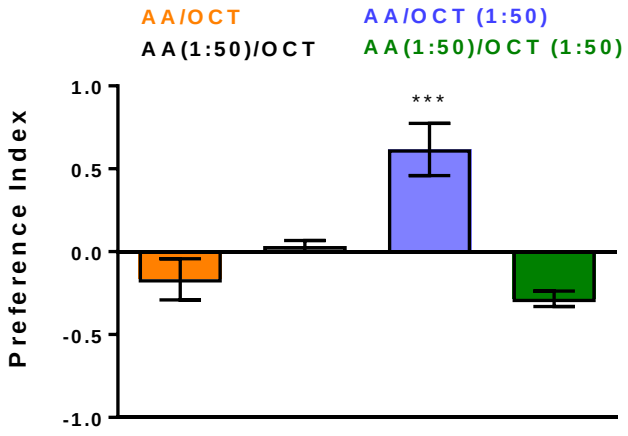

Figure 4. Odor preference in Drosophila larvae. Larvae placed in the center were given a choice between odorants n-amyl acetate or octanol that was diluted or used without dilution. Positive preference indicates attraction to n-amyl acetate, while negative preference index indicated aversion to n-amyl acetate. Groups were compared by Kruskal-Wallis tests followed by post-hoc correction with Dunn's multiple comparisons (n=10).

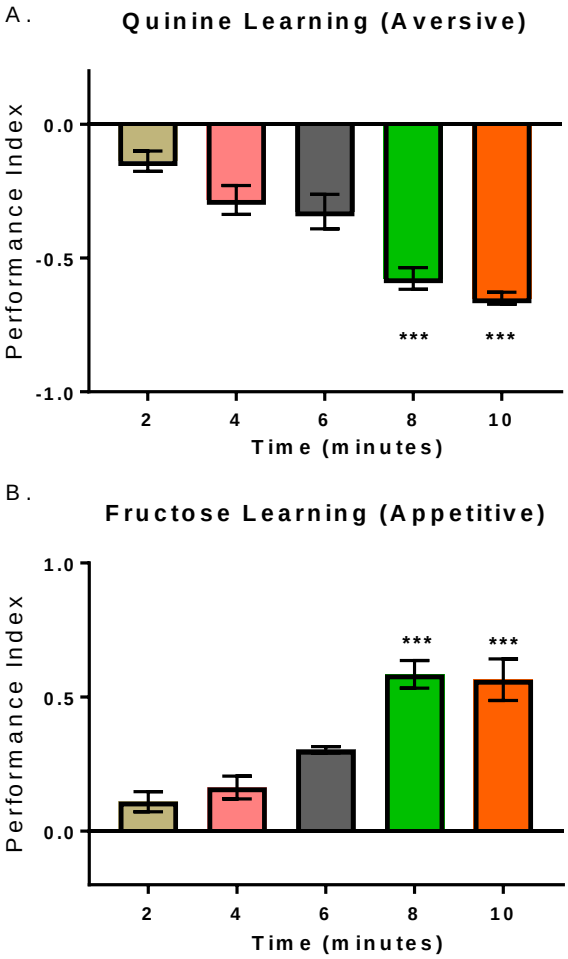

Figure 5. Performance index 2,4,6,8 and 10 minutes post-training. Negative preference (A) indicates aversion to odor associated with quinine, while, positive (B) preference indicates attraction to odor associated with fructose. Memory performance was significantly different at 10 minutes after training indicating that a strong memory of the punished odor can be formed as early as 8-10 mins. Groups were compared by Kruskal-Wallis tests with Dunn's multiple comparisons (n=12).

Single-gene mutational analysis of appetitive learning: One of the advantages of using *Drosophila* as an experimental system is the ability to study the genetic basis of behavior. We tested a deletion mutant syn97CS and wild type CS flies. We found that the synapsin mutant (syn97CS) has significantly reduced appetitive memory score at 10 minutes as compared to wild type flies. Since, the syn97CS flies had similar gustatory preference to controls, we concluded that these mutants have a defect in associative learning and not naïve odor or sugar preference (see Figure 6).

These studies can be expanded to test other mutants targeting genes involved in synaptic vesicle release and plasticity to demonstrate the importance of synaptic proteins in memory formation (Figure 6). Non-parametric analyses were employed throughout (Kruskal–Wallis tests for comparisons across multiple-groups, Mann–Whitney U-tests for two-group comparisons). Significance is inferred if $P<0.05$. N is typically 10-12 trials and all experimental data collected by undergraduate students.

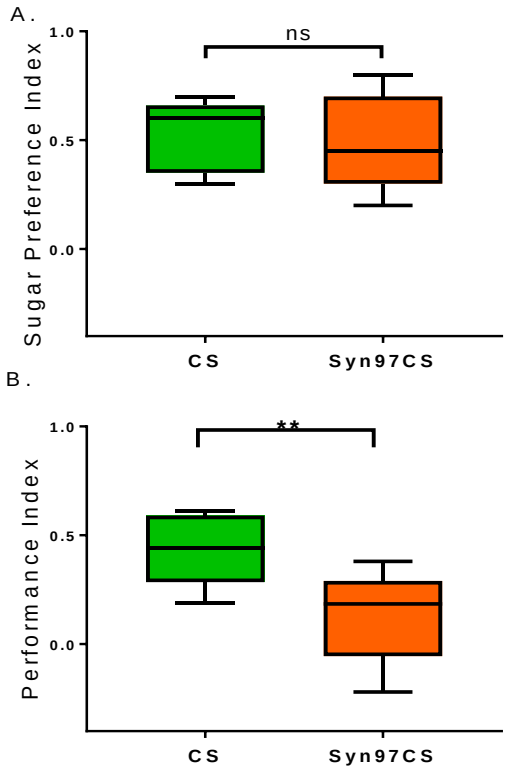

Figure 6. Synapsin mutation impairs associative appetitive memory. *(A)* Sugar/Fructose preference of wild-type and Syn97CS flies was measured using the gustatory assays described in Figure 1 and 3 (N=6). *(B)* Performance index 10 minutes post-training (appetitive conditioning) in CS (wild type) and synapsin mutant (Syn97CS). Two groups were compared by a Mann-Whitney U test (n=11, p<001).

DISCUSSION

Associative learning is an important biological function for navigating various stimuli in an organism's environment. Experimental demonstrations or laboratory modules covering learning experiments are often difficult to conduct without special equipment and expertise.

Here, we have adapted an array of chemosensory and learning assays using *Drosophila* larvae that are routinely performed in research laboratories (Gerber et al., 2009; Schleyer et al., 2011; Gerber et al., 2013; Rohwedder et al., 2016). We also describe concrete experiments for longer projects that include but are not limited to silencing and activating specific neuronal classes and targeting conserved neuromodulator systems and neurons within the mushroom body of the fly brain (a region implicated in learning and memory formation (Zars et al., 2000; Heisenberg, 2003).

These assays are simple and robust enough to be conducted in a regular classroom without laboratory equipment making them ideal for programs lacking a teaching lab (Figure 7). The cost of all module is roughly

Figure 7. Students conducting the chemosensory and learning assays in a classroom. Students were seniors in the Behavioral neuroscience major program at the University of San Diego. The activity was performed in pairs and students shared data via Google spreadsheets.

$10-15/student and can be further brought down as the petri plates and fly vials can be reused. Student also get an opportunity to collect their own data, work collaboratively in groups and share data with other groups in making graphs and conducting statistical analysis.

As part of reporting these results students submitted a laboratory report that included an introduction, materials and methods, results and conclusion section. A sample laboratory report is attached as Supplementary Material. In Fall 2016 iteration of this module students were asked to address what they learned from this module.

Eight out of twelve students reported that they had not thought of larvae as a model for behavioral studies and enjoyed working with larvae as a model for associative learning. A few students also noted that performance index should be tested for longer durations to see if larvae have long term memory. Most students agreed that these were not a complicated set of assays and yet they were able to observe instinctive behaviors and learning functions that are common in many organisms, including vertebrates.

Students also noticed the variability in behavioral data when they entered their group's results on the Google spreadsheet and identified multiple reasons for these variations including: handling of larvae between training trials, cleaning food substrate from the larvae body surface to possible errors in pipetting/diluting odorants. Students also appreciated the number of key concepts covered in neuroscience in these modules including gustatory preference, evolution of sugar attraction and bitter avoidance, odor discrimination and associative learning.

One group also noted that there might be many environmental parameters such as proximity to windows and mechanical disturbances that may contribute to difference between performance indices between groups. During the post-experiment discussion all students unanimously agreed that a bigger sample size and more experience with handling larvae would produce more reliable results that matched the numbers in published findings. We also discussed different ways of graphing these data and how statistical tests are chosen. Based on these discussions and questions it was evident that students showed a high level of engagement with the data analysis process.

In previous semesters, a behavioral data set acquired in my (DS) laboratory (Sitaraman et al., 2015) was given to students for graphing and statistical measures and students did not question the data acquisition or discuss reasons for possible variations between trials.

Four out of twelve students also expressed interest in testing additional mutants to study the genetic basis of behavior. Three of those students worked additional hours in the lab as part of independent study (NEUR 496) to complete many of the control experiments and data sets reported in this paper. These three students are authors of this manuscript and were senior undergraduate students at the time of data collection (Spring 2017). While variability between experimental trials is common in behavioral experiments, students can only appreciate this aspect of research when they set up their own experiments and acquire their own data.

Drosophila larvae are a powerful system to study the genetic and neural basis of learning and memory formation, thereby providing enough flexibility for open ended semester long research projects. Taken together, the suite of experiments reported here present concrete and flexible projects that can easily be integrated in lower-, upper-division lab experiments or outreach activities.

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