Supplementary Material for Aversive and Appetitive Learning in Drosophila Larvae: A Simple and Powerful Suite of Laboratory Modules for Classroom or Open-ended Research Projects by Pavin et al., J Undergrad Neurosci Educ, 16(2):A177-A185

Olfactory Learning in Drosophila larvae

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Abstract:

One of the main goals in neuroscience is to demonstrate how observable animal behaviors can be predicted from complex neurological processes. It is commonly understood that genetic factors influence human behavior, but how can a scientist explain how these genes interact with countless environmental stimuli to produce behavior? The complexity of the human brain would make it impossible to determine how each structure influences behavior, so attention was brought to simpler model organisms first. Model organisms typically express some complex neural structures within a simpler, more easily understood brain. *Drosophila melanogaster*, for example, uses olfactory systems that are strikingly similar to those of humans and other mammals. Eventually mapping out the structures and functions of the insect olfactory system will enable conceptualization of conserved mechanisms underlying learned and innate behaviors. By associating various odors with food reinforcement in a classically conditioned learning paradigm we will demonstrate how the absence of these structures and/or biological processes alters behavior. Larvae mutated against expression of specific genes involved in synaptic plasticity will be tested according to the same learning paradigm as genotypic controls and wild type *Drosophila* larvae. Observable behavioral differences between mutant and wild type groups will be presented to elucidate the role of synaptic plasticity in adaptive behavior.

Introduction

An organism's ability to perceive and interpret various environmental cues is important for guiding behavior. Associative learning is understood as retrospectively changing the causal status of a cue through previous learning episodes that do not directly involve the cue. In this respect, associative learning allows an organism to use past experiences to modify its behavioral response to new environmental cues. By forming associations between past experiences and an associated stimulus, an organism's interpretation of the causal status of novel environmental cues will be influenced by simultaneous sensation of it with the previously associated stimulus.

Olfactory and gustatory sensation systems have been elucidated as distinct and separate neurological pathways. When Drosophila experience gustatory and olfactory stimuli, different biochemical pathways are activated (Schroll et al., 2006). Olfactory stimuli evoke a calcium influx, signaling calmodulin activation in the MB. Gustatory reinforcers, on the other hand, bind to aminergic neuron receptors, resulting in activation of G-protein coupled amine receptors in the MB. Only simultaneous activation of gustatory reinforcement systems, denoted by GPCR activation, and olfactory stimuli, denoted by increased calmodulin levels, results in a substantial increase in adenylyl cyclase (AC) concentration in the MB (Michels et al., 2011; Saumweber et al., 2011). AC is integral to the cAMP signaling pathway, functioning as a molecular coincidence detector between separate neural events. When these neural events are represented by temporally close input signals that are spatially distributed, AC is necessary to form an association between the separate neural events (Gervasi et al., 2010). By signaling the cAMP-driven activation of kinases involved in the phosphorylation of synapsin proteins,

increased levels of AC cause the synaptic environment between these sensory pathways and behavior-guiding output neurons to become more "plastic." This plasticity allows novel synapses to form, allowing neuromodulator neurons to strengthen or weaken their synaptic complexes and alter the integration of sensory information. As a result, the collective output from afferent sensory neurons, such as olfactory neurons, can be modulated by the AC-dependent synaptic plasticity of aminergic neuromodulator neurons to elicit different responses. Since these output neurons are involved in guiding behavioral responses, an organism can use these synaptic impressions of past experiences to modulate response to novel environmental cues.

In studies of *Drosophila* melanogaster, associative learning is typically examined in the context of aversive or appetitive associations. By expressing light-activated channelrhodopsin in aminergic neuron subtypes, a differentiation between aversive and appetitive associations was made as clearly as the type of neuromodulator neuron activated in a synapse (Schroll et al., 2006). Dopamine activation elicited aversive associations with odors, while octopamine activation elicited appetitive responses to odors. This demonstrates that associative learning may be established through an antagonistic modulatory system, where the neuromodulators' strength is influenced by the synaptic environment that results from previous sensory experiences.

When the gene sequence for one of the proteins involved in MB synaptic plasticity, synapsin, was identified, it could be targeted for its role in associative learning. Drosophila mutagenesis removed the gene sequence promoter region, effectively stopping expression of synapsin proteins (Michels et al., 2005; Akbergenova and Bykhovskaia, 2007; Gerber and Stocker, 2007). When synapsin proteins were expressed

in every brain region besides the MB, the deficit in associative learning remained. This drew a correlation between MB expression of the gene sequence encoding synapsin and associative learning. Without proper expression of synapsin proteins, AC's ability to act as a coincidence detector between two associated stimuli is limited. Electron microscopy of mutant Drosophila motoneurons revealed a dispersal of neurotransmitter vesicles that was only phenocopied through exogenous activation of the motoneuron. Since synapsin proteins facilitate vesicular fusion with the cell membrane, and subsequent release of neurotransmitters, a lack of these proteins would result in a dispersal of synaptic vesicles within the bouton. Without the synapsin-guided release of the vesicular neurotransmitters, the adenylyl cyclase-signaling cascade cannot cross the synapse and alter the presence of neuromodulators. Much like the lack of synapsin-bound vesicles typical of the refractory period after an action potential, mutant motoneurons were unable to properly aggregate vesicles to release neurotransmitters. Without these chemical signalers, the neuron is incapable forming some plastic synapses necessary for modifying neuromodulatory input.

The aim of this study is to establish an associative learning paradigm that can be used to evaluate the effect of gene mutations on associative learning. By, first, establishing a systematic procedure where associative memories are consistently established in wild-type *Drosophila* and, second, using this procedure on mutant *Drosophila* strains, the effect of a single mutation on associative learning can be isolated. When novel gene mutations produce significant differences on this paradigm, the gene's function can be implicated as involved in associative learning. This is important because many homologies exist between the Drosophila and human genomes, allowancing an

opportunity to study the behavioral effects of conserved gene sequences existing in the human genome.Examining the chemical structure of synapsin proteins in Drosophila and humans, 50% of the overall identity and 89% of the amino acids were conserved in the N-terminal A domain and the actin and vesicle-binding C domain (Nuwal et al., 2011)). As a result, the similarities between gene sequences in Drosophila and humans will lay the foundation for comparative analysis of their influences on behavior.

Drosophila larvae possess complex chemosensory systems allow the perception of environmental orders and tastants. Although these complex systems have been a topic of study, the connection between olfactory and gustatory sensation is not well understood (Schroll, 2006). These chemosensory systems are complex and difficult to interpret in adult *Drosophila*, so the relatively simple framework of the larval brain offers a context to elucidate the role of these chemosensory systems in associative learning. Even though the developing larvae may possess simple sensory systems, they still demonstrate an innate preference for odors and tastes that can be tested in a laboratory setting. We examined these preferences through odor and taste learning and choose assays, determining concentrations at which odors demonstrated no preference. By pairing these experimentally established "neutral" odors with punishment and reward conditions, an association between olfactory sensation and gustatory stimuli could be established. This association can then be tested in performance assays to suggest the presence of associative learning and memory potentiation over time. Performing these assays with wild type and transgenic mutant Drosophila will provide the opportunity to identify genetic influences on associative learning.

Methods

Materials

- Fructose, Agarose, and Quinine
- Petri dishes, perforated petri lids
- N-amyl acetate (AA), 1-octanol (Oct) and paraffin oil
- Filter paper and double-sided tape, pipette
- Spatula, wash boats, paintbrush, stop watch
- Feeding stage (5 day-old) larvae and plastic vial

Substrate	Concentration	Solvent	Preference
Agarose	1%	Water	Neutral
Fructose	2.0 M	1% Agarose	Positive
Quinine	0.5 mM	1% Agarose	Negative

Odor	Dilution	Solvent	Preference
AA (N-amyl acetate)	1:50	Paraffin oil	Neutral
Oct (1-octanol)	Pure	None	Neutral

Preference Index

Preference for X side = [(# larvae on X side) - (# larvae on opposing side)] / [# larvae total]

Examples…

Performance Index for AA paired with Fructose

$PI_{AA+/OCT}$ = (larvae on AA side - larvae on Oct side) / total number of larvae

Performance Index for Oct paired with Fructose

$PI_{AA/OCT+}$ = (larvae on Oct side - larvae on AA side) / total number of larvae

Performance Index for AA paired with Oct

$PI_{AA-/OCT}$ = (larvae on AA side - larvae on Oct side) / total number of larvae

Preference Index for Oct paired with Fructose

$PI_{AA/OCT-}$ = (larvae on Oct side - larvae on AA side) / total number of larvae

Notation: (+): pairing with Fructose (reward, positive, & appetitive association)
(**-**) : represents pairing with Quinine (punishment association)
(**AA**) : pairing with N-amyl acetate (odor)
(**Oct**) : pairing with 1-octanol (odor)

Collection of Larvae

We collected 5-day-old larvae to ensure they remain in feeding stages. Using a spatula, larvae were scooped from the feeding container and dispersed on a wash boat. Using a moist paintbrush, each larva is extracted from the food and moved to a clean

wash boat. Washing the larvae with distilled water, this process is repeated until no excess food remains. When 30 larvae are collected, they are transferred to a plastic vial containing 5 mL distilled water.

Innate Gustatory Preference

Preparing petri dishes. For this assay, petri dishes are made with different substrates on either half. Petri dishes are first filled with 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 that is mixed with fructose or quinine. These substances have been reported as appetitive or aversive gustatory stimuli, respectively, in Drosophila larvae gustation. 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.

Innate Preference. Using a paintbrush, larvae are removed from the plastic vial and placed on a water boat. Ensuring that no excess water is transferred with the larvae, position all 30 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, is 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. After data was collected, preference index (PI) values were calculated for each assay and averaged across conditions for fructose or quinine. In order to calculate the preference index, the number of larvae found on the fructose or quinine side is subtracted the number of larvae found on the opposing, agarose-containing side and divided by the total number of larvae in the petri dish. The resulting value is then divided by the total number of larvae in the dish.

This will yield a PI value for fructose or quinine, so PI scores are averaged across conditions that use either fructose or quinine. A T-test is performed on the resultant PI values to determine if there is a significant difference between innate fructose and quinine preferences.

Innate Olfactory Preference

Innate Preference. Two pieces of double-sided tape are positioned on opposing sides of the interior surface of a perforated petri lid. Strips of filter paper are placed on either piece of tape. Oct is used to drench one piece of filter paper, while AA is used for the opposing side. After 30 larvae are transferred from a vial to an agarose-containing petri dish, the lid is closed for five minutes. The entire substrate lining the petri dish is agarose in this assay, so the odors should present the only variable between opposing sides. When five minutes has past, larvae are counted to calculate PI values for either side of the petri dish. PI values for these assays will reflect odor preference, providing an opportunity to identify the concentration most likely to elicit a “neutral” preference. The four AA and Oct dilution conditions that will be used for this assay are 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; PI values for AA were generated in each trial, while the PI value for each condition was generated as an average of the three trials. The distinction between positive and negative PI values is the preference towards AA and Oct, respectively, at the given condition’s odor concentration.

Associative Learning:

Training. 30 larvae are removed from a plastic vial and positioned along the

midline of an agarose plate. A perforated lid with filter paper taped along the inner surface of either side is placed on the agarose petri dish. After five minutes, the larvae are transferred to another petri dish containing fructose and covered by another perforated petri lid. After five minutes, the larvae are transferred back to the original agarose dish to repeat the training procedure two more times. Since different odors are used to soak the filter paper covering agarose or fructose substrates, the larvae only experience one substrate for each odor. Fructose was identified as a positive, reward, or appetitive stimulus, so odors associated with fructose will carry the following notation: +. The entire procedure is completed under two conditions as follows: Oct paired with agarose (Oct) and AA paired with fructose (AA+) or AA paired with agarose (AA) and Oct paired with fructose (Oct+).

The entire training process is repeated with quinine substituted for fructose. Quinine has been identified as a negatively reinforcing, aversive stimulus, so odors associated with quinine will carry the following notation: -. As a result, two more groups will be trained according to the following conditions: Oct paired with agarose (Oct) and AA paired with quinine (AA-) or AA paired with agarose (AA) and Oct paired with quinine (Oct-).

Control Tests. Immediately after training is completed, the larvae are transferred to the midline of a new agarose dish; the conditions for testing are exactly the same as innate odor preference conditions. The perforated lid containing AA and Oct is placed on the petri dish and the larvae on either side are counted in two-minute intervals for ten minutes. At ten minutes, the larvae are counted, collected, and removed from the petri dish. The testing procedure is repeated for each of the four training groups (AA+, Oct+,

AA-, Oct-)

Performance Index values are calculated according to the same procedure for calculating preference index values; the only difference is lies in the associated experience that may influence performance indices.

Although there was no significant difference between each of the dilutions via ANOVA, we chose the dilution that was closest to a baseline of neutral preference; because of this, we averaged each of the two-minute interval values for fructose across both the amyl acetate and octanol conditions.

In order to evaluate the effects of reward and punishment training conditions on testing preference, PI scores are averaged across trials for reward or punishment conditions. Thus, PI values for each 2-minute interval were averaged for AA+ and Oct+ conditions or AA- and Oct- to yield reward or punishment PI values, respectively. Now, each of the two-minute interval PI values for either reward or punishment performance indices must be compared to a baseline preference index value through a T-test. Since ANOVA demonstrated no significant difference between preference indices for each of the odor concentrations tested for innate olfactory preference, the odor concentrations (AA 1:50 and Oct) that yielded the lowest absolute PI value were used as a baseline (Tables 2a-2d). These concentrations resulted in the lowest magnitude of preference towards one odor over the other, so it best represents a “control” value. Since the odor concentrations used for a baseline value are also used in each of the testing assays, T-tests will compare the baseline preference index with trained performance index values. This way, any innate preferences from the odor concentrations will be present in both the baseline and testing PI values. As a result, the remaining variable between the conditions

is limited to associative learning. Performance index values that significantly differ from the baseline preference index values will reflect the influence of associative learning from previous exposure to punishment or reward conditioning.

Mutant Tests. Assays involving mutant larvae strains will follow the same procedures for control tests. The only difference is the type of larvae used in the experiment.

Results

Table 1a: Innate Taste Preference (Fructose)

Trial	#Larvae (Fructose)	#Larvae (Agarose)	# Larvae (Total)	Preference Index
1	27	3	30	0.8
2	18	5	23	0.57
3	19	5	24	0.58
4	16	4	20	0.6
5	14	6	20	0.4
6	15	5	20	0.5

Mean Preference Index: 0.575

In every trial, more larvae migrate to the fructose than the agarose side of the petri dish. This reflects a behavioral trend in search of fructose.

Table 1b: Innate Taste Preference (Quinine)

Trial	# Larvae (.5 mM Quinine)	# Larvae (1% Agarose)	# Larvae Total	Preference Index
1	8	31	39	-0.59
2	13	23	36	-0.28
3	9	22	31	-0.42
4	7	24	31	-0.55
5	11	21	32	-0.31
6	7	23	30	-0.53

Mean Preference Index: -0.445

More larvae migrate to the agarose side of the petri dish in every trial. This reflects a

behavioral trend in avoidance of quinine. A T-test between the averaged PI values for quinine or fructose demonstrated that there is a significant difference between the conditions (123). Since the only variable between the two conditions is the type of substrate, it can be assumed that the positive fructose-associated PI values and negative quinine-associated PI values reflect appetitive or aversive qualities, respectively, ine ach substrate.

Figure 1 Performance Index for innate Quinine and Fructose Preference

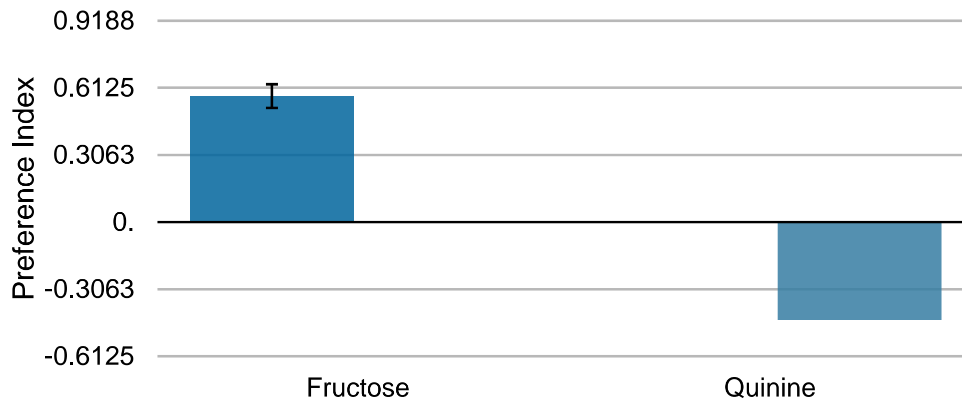


Table 2a: Innate Odor Preference (AA / Oct)

Trial	#Larvae (AA)	#Larvae (Oct)	# Larvae (Total)	Preference Index
1	11	20	31	-0.29
2	10	18	28	-0.29
3	13	11	24	0.08

Mean Preference Index: -0.17

Table 2b: Innate Odor Preference (AA 1:50 / Oct)

Trial	# Larvae (AA)	#Larvae (Oct)	# Larvae (Total)	Preference Index
1	10	10	20	0
2	10	10	20	0
3	11	9	20	0.1

Mean Preference Index: 0.03

Compared with the other dilutions in Tables 2a – 2d, this dilution yields the smallest preference index. This PI value can be used as a "control" for demonstrating the effect of associative learning. Rather than considering the effects of both innate odor preference and associative learning on performance index values, the innate odor preference is accounted for. As a result, the only variable that should performance indices associative learning.

Table 2c: Innate Odor Preference (AA / Oct 1:50)

Trial	# Larvae (AA)	#Larvae (Oct)	# Larvae (Total)	Preference Index
1	29	1	30	0.93
2	9	16	25	-0.28
3	18	7	25	0.44

Mean Preference Index: 0.36

Table 2d: Innate Odor Preference (AA 1:50 / Oct 1:50)

Trial	# Larvae (AA)	#Larvae (Oct)	# Larvae (Total)	Preference Index
1	9	18	27	-0.33
2	7	14	21	-0.33
3	11	16	27	-0.19

Mean Preference Index: -0.28

Table 3a: Reward Performance Indices (Fructose: AA+, Oct+)

Time (min)	AA+ / Oct	AA / Oct +	AA / Oct+	P-Value (T-Test of Averaged PI Values and Baseline)
2	0.08	0.36	0.25	0.1968
4	0.28	0.28	0.27	**> 0.0001**
6	0.14	0.44	0.29	0.0505
8	0.31	0.5	0.44	**0.0112**
10	0.00	0.44	0.25	0.2097

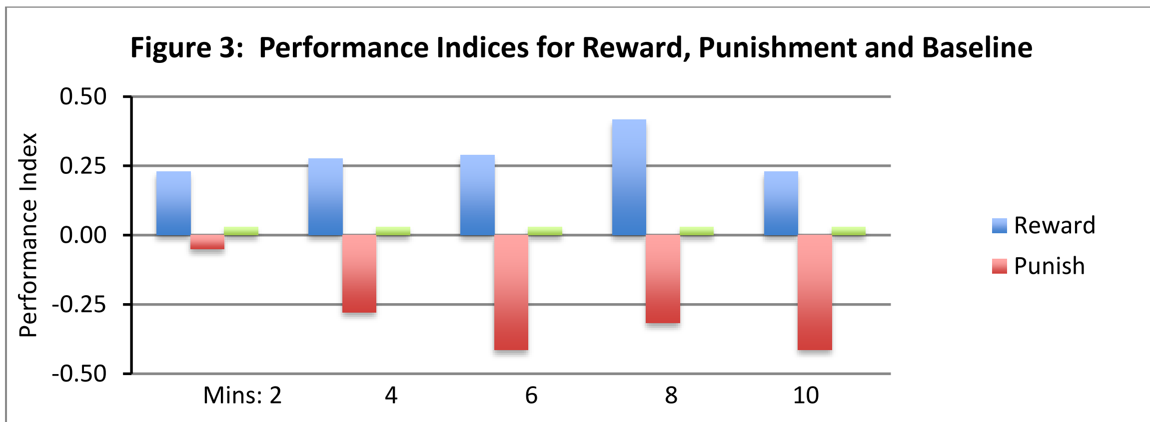
At four and eight seconds, a significant difference exists between the baseline preference index and the average of positively reinforced performance indices. In every time interval other than 4 seconds, AA+ results in a lower PI score than Oct+

conditioning. This may suggest that Oct is more easily associated with positive reinforcement or AA is innately less preferred.

Table 3b: Punishment Performance Indices (Quinine AA-, Oct-)

Time (min)	AA- / Oct	AA / Oct -	P-Value (T-Test of Averaged PI Values and Baseline)
2	-0.1	0.00	0.2394
4	-0.38	-0.18	0.5759
6	-0.61	-0.22	0.5159
8	-0.44	-0.19	0.5545
10	-0.57	-0.26	0.5890

There are no time intervals where a significant difference exists between baseline preference index and the average of negatively reinforced performance indices. An interesting observation is that the PI values for AA- are more than twice the magnitude of PI values for Oct-. Since the PI values are negative, which suggests a preference against the negatively reinforced odor, the more negative AA- PI values suggest that different odors may produce different outcomes. AA could either be more easily associated with negative stimuli or Oct could be innately more preferred.



This figure illustrates the Reward and punishment-associated PI values recorded in two-minute intervals over ten seconds. Regardless of a statistical significance only

existing in the different between baseline and reward PI values at four and eight minutes, the overall trends suggest a negative or positive influence from punishment or reward conditioning, respectively. According to the trend, it appears that the effect of associative learning is the strongest from between four and eight seconds.

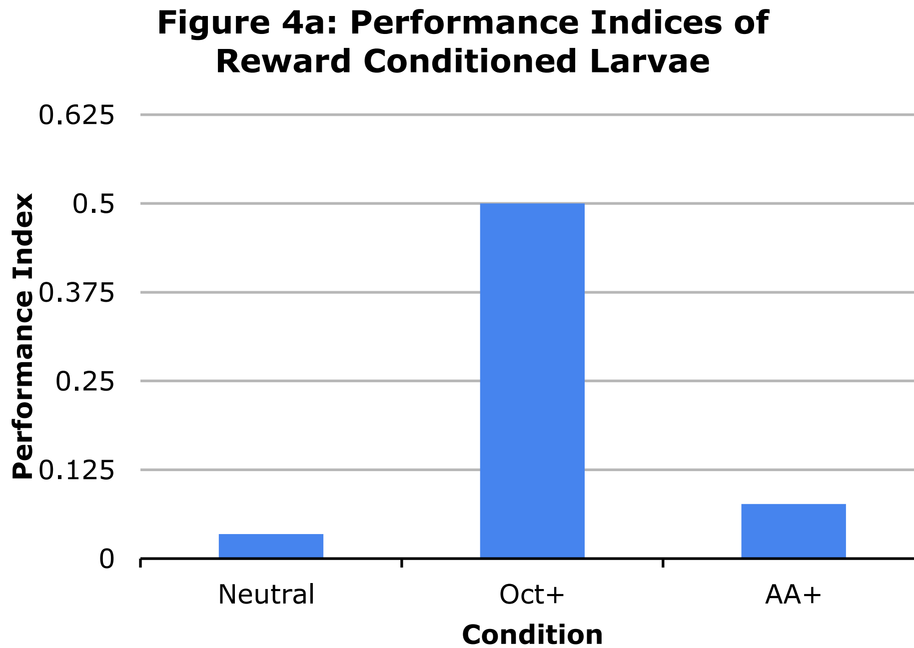


Figure 4a shows the calculated performance indices for additional reward training assays. The neural, baseline PI is 0.0345. The reward conditions, Oct+ and AA+, resulted in PI values of 0.5 and 0.077, respectively. PI values for Oct+ and AA+ were both more positive than the baseline, so associative learning may have influenced the performance indices.

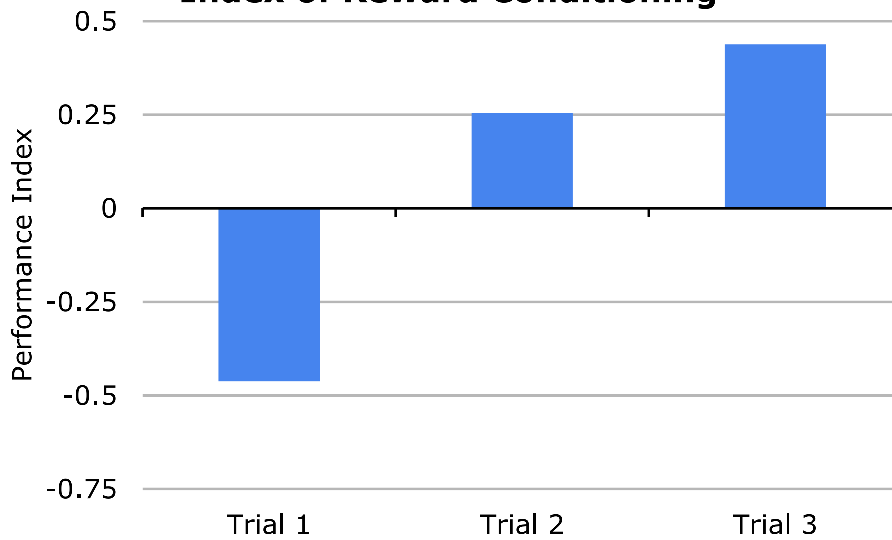
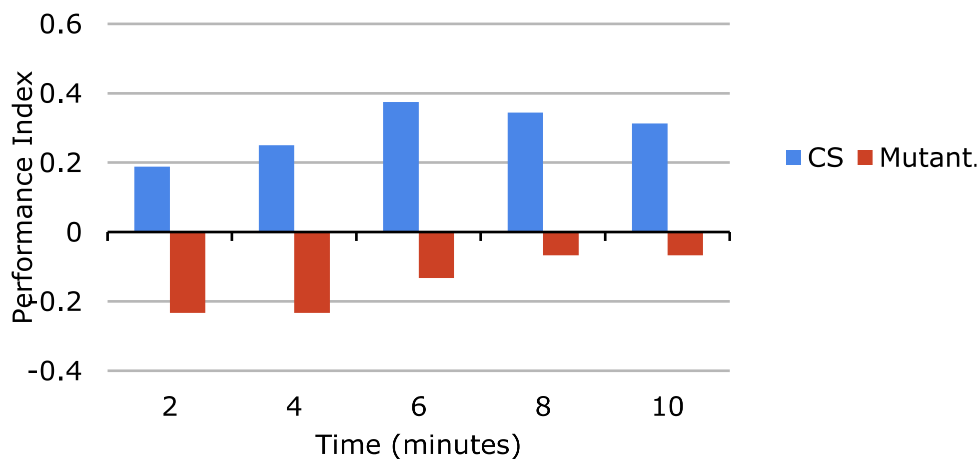
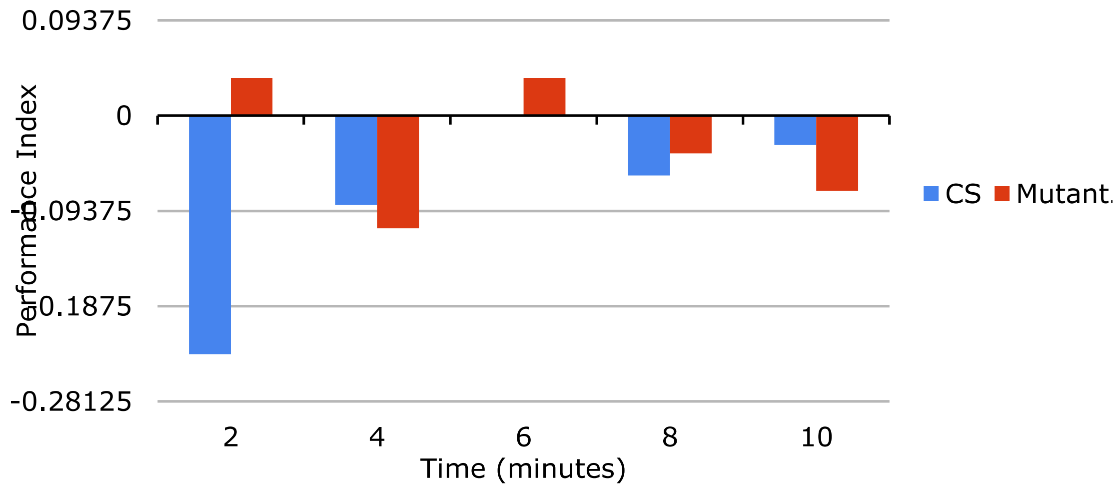


Figure 4b illustrates the three trials used to average the PI value for AA+. The first trial may have resulted from experimental flaws, which would negatively skew the overall average for AA+ PI.



One explanation for these values is that Oct may be an innately appetitive odor. If the odor's appetitive qualities are stronger than the negative associations made through punishment pairing.



This graph illustrates an attempt to positively reinforce AA with fructose. If Oct is an innately appetitive odor, then producing positive PI values for the AA+ condition would demonstrate associative learning. By positively reinforcing AA with reward, the PI would reflect a preference towards AA+ over Oct. Interestingly; the control group produced sporadic, yet negative, AA+ PI values. The mutant group shifted between positive and negative PI values, demonstrating a more random trend.

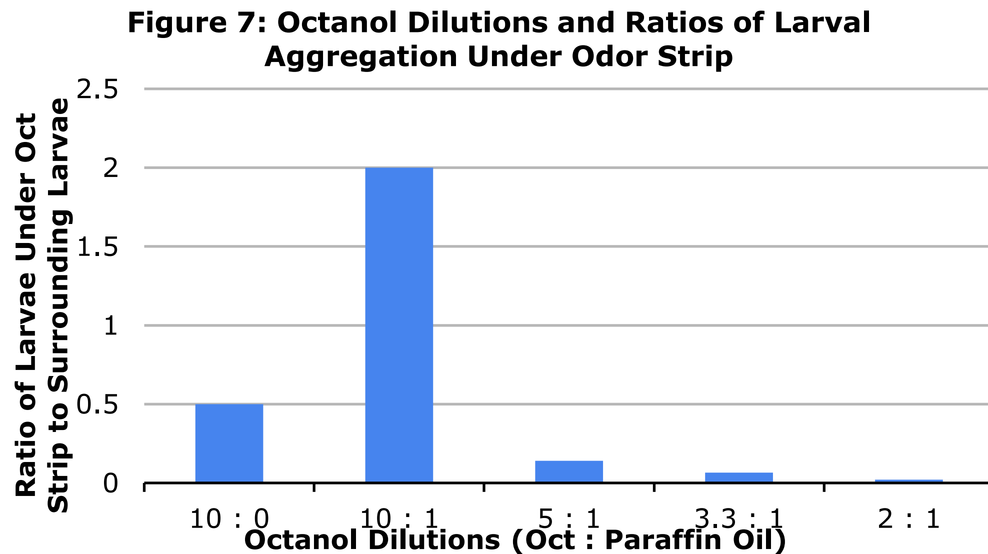
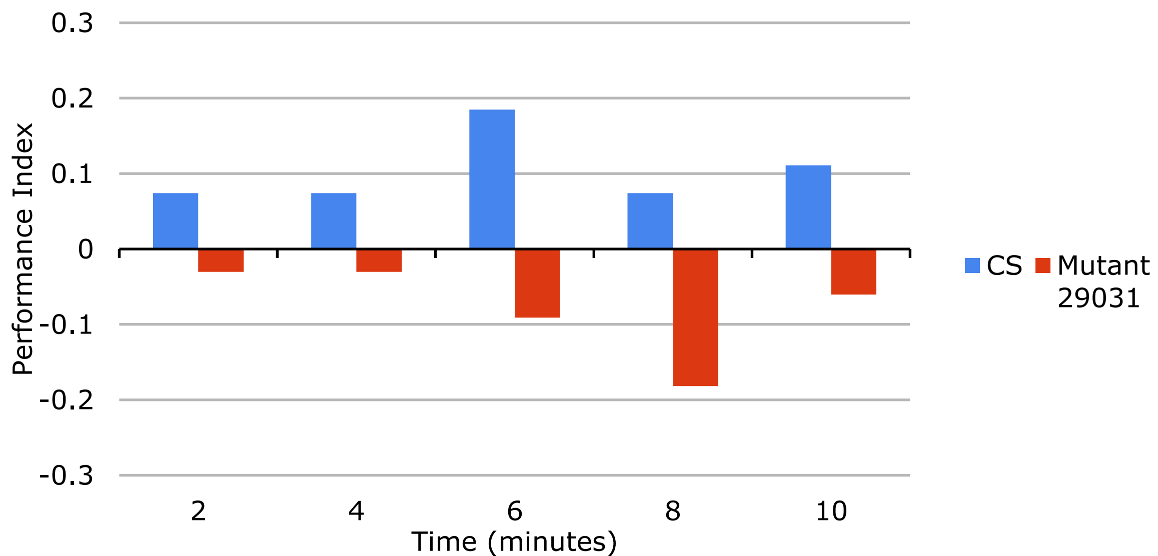


Figure 7 illustrates the relative number of larvae attracted to Oct at different dilutions. Oct and AA can be either aversive or appetitive at different concentrations, so a less appetitive dilution could exist. The larvae aggregating, meaning the formation of a tight, clustered ball, underneath the odor-drenched filter paper were recorded, as well as the remaining larvae on the petri dish, to illustrate the innate preference for Oct at different dilutions. In the first dilution (10:0) of pure Oct, one third of the overall larvae travelled to the small region of agarose substrate directly under the Oct odor paper. In the second dilution (10:1) of Oct in paraffin oil, two thirds of the overall larvae travelled to the small region of subsrate directly under the Oct odor paper. The final dilutions (5:1, 3.3:1, and 2:1) of Oct in paraffin oil resulted in much less larvae aggregating under the odor. In the 3.3:1 dilution, the larvae appeared evenly distributed across the substrate. Since the Oct needs to be sensed by the larvae for associations to occur with other stimuli, dilutions of 2:1 and lower were avoided. By using the dilution with no apparent preference towards the odor at the highest concentration of Oct, the likelihood for the larvae to sense the odor is maximized.



This figure shows that control group larvae yielded positive PI values for AA+ conditioning if a 3.3:1 dilution of Oct in paraffin oil is used. This means that the reward conditioning may have caused the formation of positive associations between AA and fructose. Mutant strain 29031 did not yield positive PI values at any time interval.

Discussion

This experiment was successful in demonstrating that Drosophila Melanogaster larvae are capable of associative learning. Table 1a suggests an overall preference index of 0.575 for fructose when presented with fructose and agarose. Table 1b suggests a preference index of -0.445 for quinine when presented with quinine and agarose. These results suggest an innate preference towards fructose and against quinine in Drosophila larvae. Thus, fructose can be identified as an appetitive stimulus (+) and quinine as an aversive stimulus (-). Table 3 reflects a preference index of -0.17 towards AA when presented with undiluted AA and Oct (AA / Oct). Table 4 shows a preference index of 0.03 towards AA when presented with diluted AA and undiluted Oct (AA 1:50 / Oct).

Table 5 shows the mean preference index of 0.36 towards AA when presented with undiluted AA and diluted Oct (AA / Oct 1:50). Table 6 shows a mean preference index of -0.28 when presented with both diluted AA and diluted Oct (AA 1:50 / Oct 1:50). These results produced an ANOVA with a P-value of 0.158, shown in Figure 3. This reflects no significant difference between the PI values for any of the four odor dilution conditions, yet the (AA 1:50 / Oct) dilution Table 2b results in a PI value of 0.033 (Table 2b). This value is closest to 0.00, so it will be used as a baseline (Tables 2a-d). The same dilutions that produced this baseline value are also used in the testing assays, so using the innate PI value for this dilution will account for the effect of odor concentrations on the trained PI values. The remaining manipulated variable between the innate PI and trained PI conditions is the presence of associative learning. As a result, trained PI values that differ significantly from the innate "baseline" PI values should implicate the presence of associative learning. These values are identified with P-scores under 0.05 via T-tests of innate and trained PI values.

Performing assays with these concentrations, Figures 3a-b demonstrate the positive or negative preferences, respectively, towards odors associated with reward or punishment conditioning, respectively. These odors have been paired with either fructose or quinine to elicit a reward or punishment condition, respectively. Isolating 2-minute intervals as individual graphs, T-tests were performed to calculate a P-value and determine if the behavioral response associated with a condition, indicated in the PI of that condition, is significantly different from the baseline preference indices determined in Table 4. In trials with fructose reward pairing shown in Figure 3a (AA+ / Oct PI values averaged with AA / Oct + PI values in each trial), time intervals at 2, 6, and 10 minutes

produced P-values of 0.1968, 0.0505, and 0.2097, respectively. These suggest no significant difference in performance indexes at these time intervals. At six minutes, a P-value of 0.055 demonstrated that there was almost a significant difference for that interval. The remaining p-values on Figure 3a are 0.0001 and 0.0112, indicating a significant difference between PI values at four-minute and eight-minute time intervals and baseline PI. This suggests the presence of associative learning around four or eight minutes into the testing assays. In trials with quinine punishment pairing (AA- / Oct PI values averaged with AA / Oct- PI values in each trial), no significant difference exists between trained PI values and baseline values. Figure 3b organizes the P-values for each time interval, yielding no values that reflect a significant difference between punishment conditioned PI values and the baseline PI value. These values suggest that negative, punishment assays may not be a preferred context for associative learning in Drosophila larvae. Drosophila larvae are, however, capable of associative learning in positive, appetitive conditioning assays, bridging the appetitive qualities of fructose to odorants previously determined as neutral or non-appetitive. The fact that there was less of a significance correlating performance indices for reward and baseline associated odors may shed light to the memory duration of Drosophila. It appears that the association between varying stimuli may take about four minutes, and that the association, or memory, diminishes after eight minutes.

In order to further develop and perfect this paradigm, more trials were performed with associative learning assays. Rather than averaging PI values across all reward conditions (AA+ and Oct+), PI values were averaged for each odor (AA or Oct). The previous results in Table 3a suggest that reward conditioning is more likely to produce

associative learning. A new baseline value of 0.0345 was established, while AA+ and Oct+ conditions resulted in PI values of 0.5 and 0.077, respectively (Figure 4a). Oct+ produced a much higher PI value than AA+, but both conditions yielded PI values higher than the baseline. Examination of the three trials averaged for the AA+ PI value reveals an outlier on the first trial (Figure 4b). The first trial, which could have resulted from experimental error, may have negatively skewed the averaged PI value for the AA+ condition. Thus far, reward conditioning has produced performance index values that are more positive than the baseline PI value, except in Figure 4b trial one.

PI values are now calculated for each odor, similar to the above procedure, but with quinine replacing fructose. Now that enough larvae had developed for mutant larvae strains, assays were performed, first, with a control group and, second, with a mutant group. This would allow the results between the two groups to be directly compared; any experimental flaws were likely to be present in both mutant and control conditions. When PI values were calculated, Oct- trained control group larvae demonstrated a preference towards Oct- at every time interval (Figure 5). In the mutant group, however, there were negative, much smaller PI values for Oct. This trend reflects associative learning, but the magnitude of the PI values was fairly small and sporadic. Interpreting these results, it is possible that Oct possesses innately appetitive qualities. This would result in the preference indices that are skewed towards Oct, even when Oct is reinforced with punishment conditioning. Examining the reward performance indices in table 3a, every interval, aside from 4 minutes, resulted in PI values for Oct+ that are higher than PI values for AA+ conditioning. These results support the notion that undiluted Oct is an appetitive stimulus, suggesting that the difference in Oct+ and AA+ PI values resulted

from innate preferences. Examining the PI values for each time interval for punishment conditioning in Figure 3b, every PI value for Oct- is less than half of the magnitude of PI values for AA-. This demonstrates that pairing Oct with a punishment condition still results in a preference for Oct.

Oct and AA can possess aversive or appetitive qualities at different concentrations, so changing the concentration of Oct used in these assays may reduce the appetitive qualities. Figure 7 illustrates the different dilutions of Oct that were evaluated. By counting the number of larvae aggregating under the Oct-soaked filter paper, as well as the number of larvae on the remaining surface of the petri dish, ratios were made to represent larvae under the strip relative to the remaining larvae. One third of the larvae aggregated under the Oct-drenched paper with undiluted Oct, while a 10:1 dilution of Oct in paraffin oil resulted in two thirds of the larvae aggregating under the paper. 5:1, 3.3:1, and 2:1 dilutions resulted in much less larvae aggregative under the strip. For this experiment, the 3.3:1 dilution was adopted.

Using this new dilution, AA+ training will be performed again. If AA can be trained to have a positive PI value when presented with Oct, then associative learning must be occurring. Figure 8 shows the PI values for control and mutant 29031 groups in AA+ training. As a result, the control group yields positive PI values for AA+. This suggests that AA can be associated with reward conditioning. The mutant group, however, yielded negative PI values at every time interval. The influence of associative learning for the mutant group cannot be implicated. By referencing flybase.org, an online database, the effect of the mutation could be identified. Mutant strain 29031 possessed a mutation in the gene sequence encoding synapsin proteins. As was discussed in the

introduction, synapsin proteins are responsible for fusing vesicles with the cell membrane and releasing neurotransmitters into the synapse. When gustatory and olfactory systems are simultaneously activated, allowing AC to function as a coincidence detector between these separate neural events, synapsin proteins facilitate the transmission of neurotransmitters. These chemicals are integral to synaptic plasticity, so associative learning relies on the presence of synapsin proteins.

Conclusion

Associative learning is an important biological function for navigating various stimuli in an organism's environment, yet it can be tested within a laboratory setting. By establishing a learning assay, the preference towards an odor can be quantified as a PI value. Preference indices reflect an organism's innate preference towards an odor, while performance indices reflect an organism's learned preference to an odor. In order to learn this preference, the organism must form an association between the odor and another stimulus. If the stimulus elicits a preference, the preference can be associated with the simultaneously presented odor. This was performed with control groups, resulting in measured preferences that changed significantly as a result of training only in reward conditioning. Although punishment training was not successful in demonstrating a significant change in each preference, the results from reward training suggest associative learning after four minutes. Diluting Oct in paraffin oil, a new dilution of 3.3:1 was suggested to have more neutral qualities than pure Oct. Using this dilution, PI values reflected associative learning in control groups and not the mutant group. Since the mutation affects the expression of a protein involved in associative learning processes, it can be inferred that the mutation should affect associative learning. This supports the

results, as well as the hypothesis that associative learning could be established in a learning paradigm and used to evaluate the presence of associative learning in mutant strains. Using this technique, more mutant strains can be evaluated to understand which genes affect associative learning.

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