Associative learning in larval *Drosophila melanogaster:* The role of heredity in learning speed and persistence of leaning into adulthood

Jasmin Hicklen, Domonique Jones and Michelle Vieyra

University of South Carolina Aiken, 471 University Parkway, Aiken, SC 29801

Received March 29, 2010

The ability to form an association between a naturally rewarding taste stimulus and a novel odor stimulus has been observed in drosophila larvae. Studies have shown that larvae will generally learn the association after up to three stimulus pairings. Other studies have shown that drosophila adults can maintain associations learned between an electric shock and a novel odor as larvae. The purpose of this study was threefold: 1) Determine whether the ability to associate two stimuli and respond to the conditioned stimulus after a single stimulus pairing versus repeated pairings is a hereditary trait in drosophila larvae, 2) Determine whether associative learning memory carries on into adulthood by testing the adults' memory for the conditioned odor, 3) Examine whether the selective breeding of fast and slow learning larvae has an effect on memory persistence. Results indicate that learning usually occurs after one stimulus pairing, that being a fast versus slow learner may not be hereditary and that being a non-learner can be selected against. The ability to associate an odor with a taste stimulus does seem to persist into adulthood although there is little correlation with selective breeding for fast versus slow learners.

Introduction

Associative learning through the process of classical conditioning involves an unconditioned stimulus being paired with a neutral stimulus. The outcome is that the neutral stimulus will eventually become a conditioned stimulus which an animal will respond to alone just as it does to an unconditioned or natural stimulus (Dukas 1998). Associative learning is important to animals because it allows them to obtain food, avoid predators, gain social partners, and predict changes in environmental conditions (Scherer et al. 2003). Studies pertaining to associative learning have been carried out using *Drosophila melanogaster* because they represent simple models with olfactory systems comparable to that of mammals (Scherer et al. 2003; Kreher et al. 2005). Studies have shown that various substances can generate olfactory and gustatory responses in fruit fly larvae (Heimbeck et al. 1999). A recent study used olfactory and gustatory stimuli to show that associative learning can occur in the larvae of fruit flies (Scherer et al. 2003). The researchers paired an odor and a taste together so that the fruit fly larvae could learn to associate the two. One odorant (A) was paired with a positive gustatory or taste reinforcer (fructose) while another odorant (B) was paired with a negative taste reinforcer (sodium chloride and quinine), and a reciprocal training treatment was conducted on a second group of larvae (Scherer et al. 2003). The two neutral odorants used were isoamylacetate and 1-octanol (Scherer et al. 2003). After training, the larvae were individually tested to note which odor they gravitated towards without the use of a taste reinforcer, and associative learning was indicated by the differences in choice of odor between the larvae of the two treatment conditions (Scherer et al. 2003). The results of this study provided evidence of associative learning in fruit fly larvae for both treatment types (Scherer et al. 2003). A later study showed that fruit flies may take up to three conditioning trials to learn to associate a taste with an odor. Those that do not learn after three conditioning trials will not learn the association (Neuser 2004).

Additional research indicates that associative learning memory can be carried into adulthood for *D.melanogaster* (Tully et al. 1994). The study utilized an electric shock stimulus paired with an olfactory stimulus rather than a gustatory stimulus to train the fruit flies during the larval stage. The study showed that odor avoidance through a conditioned stimulus was still present once the larvae matured into adulthood (Tully et al. 1994). There have not been any studies to thoroughly test if the use of a gustatory stimulus will allow for memory persistence in adult fruit flies. Furthermore, there have been no studies to test if the associative learning process is hereditary in larvae (specifically regarding how many conditioning trials it takes to make the association), or if memory persistence into adulthood can be affected by the selective breeding of larvae.

The first objective of this experiment was to condition fruit fly larvae by pairing an olfactory stimulus with a gustatory stimulus and test for associative learning memory. The second experimental objective was to determine if the ability to associate two stimuli and respond to the conditioned stimulus after a single conditioning experience or after repeated experiences is a hereditary trait by identifying and breeding fast and slow learning larvae. The third objective was to examine if associative learning memory carries on into adulthood by testing the adults' memory for the conditioned odor. The final objective was to examine if the selective breeding of fast and slow learning larvae has an effect on memory persistence. We hypothesized that the ability to learn to associate a neutral odor to an unconditioned stimulus is hereditary as well as the number of conditioning trials it takes to make the association. Furthermore, we expect that learning will persist into adulthood, and it may be stronger in fast learners than in slow learners.

Methods

Apterous *Drosophila* larvae of unknown sex or age were used throughout the investigation. Apterous flies were chosen to aid in the transfer of adult flies in the persistence trials without the use of a sedative. The investigation was conducted on two separate colonies of *Drosophila melanogaster* to allow for statistical analysis at the conclusion of the study. The two colonies were conditioned and tested on alternate weeks. The experiment began with 60 larvae from each colony. Two sets of agar plates were prepared for the experiment. One set of plates were made with fructose in the agar mixture, while the other set of plates were made without fructose. At the beginning of each experimental day, all fruit fly larvae were taken from the fly medium and washed in nanopure water. After being washed they were put on a holding plate without fructose. The larvae were conditioned for 5 minutes on an agar dish made with Fructose as the taste stimulus with 3.0 µl of isoamylacetate placed on opposite sides of the plate in a small tube inserted into the agar. Isoamylacetate served as the neutral odor stimulus. Next, the larvae were tested on an agar plate containing no Fructose with isoamylacetate placed on one side of the plate in a small tube. The larvae were vertically aligned in the center of the plate and allowed to move freely for 1 minute. The larvae were placed back in the center of the plate and allowed to move freely a second time for 1 minute. The larvae that moved at least a quarter of the way across the plate towards the odor twice were labeled as fast learners and placed into a vial to mature and breed for the next generation. The larvae that failed this task were conditioned again for 5 minutes on the fructose plus odor plate. Once again the larvae were tested and any larvae that moved towards the odor twice were labeled as medium learners and placed in a separate breeding vial. The remaining larvae were conditioned again for 5 minutes. These were tested and the larvae that went towards the odor twice were labeled as slow learners and placed into a third vial. The larvae that did not go towards the odor twice at any time were labeled as non-learners and were disposed of. This process was repeated with 35 larvae from the fast group and 35 larvae from the slow group through 4 generations of fast and slow learners for both colonies of *Drosophila* larvae. The number of fast, medium, slow, and non-learners were counted during each trial, and the average number and standard deviation was compared across generations at the end of the experiment. A t-test was also conducted to determine whether or not the results of the experiment were significant.

All adult fruit flies from the parental generation through the F4 generation were tested to see if associative learning memory is sustained in adulthood, and whether learning speed and/or selective breeding is correlated with sustained memory. Because all available adults were tested sample sizes varied for each group. Because there were more fast learner larvae identified and continued into the next generation, sample sizes for adult fast learners ranged from 23-51 across the generations while sample size for adult slow learners ranged from 12-21 across the generations. Nanopure water was used as a negative control and apple cider vinegar was used as a positive control to test the adult fruit flies' memory for attraction to isoamylacetate. A one foot long testing tube was used to test the adults. The testing tube was divided into three sections; A, B, and C. To test for associative learning memory, a group of fruit flies were placed in the center of the tube and allowed 30 seconds to get used to their surroundings. Foam corks containing no test odor and no moisture were placed at both ends of the tube during this time period. The test odor was applied by inserting 5µL of the testing substance (water, vinegar, or isoamylacetate) onto a cotton ball attached to a foam cork on one side of the tube. Nanopure water (5µl) was inserted onto a cotton ball attached to a foam cork on the other side of the tube in order to control for moisture. Once the respective test odor and moisture were applied, 60 seconds were allowed to elapse before the odor and moisture were removed (by turning the cotton balls towards the outside of the tube). The number of flies present in the section containing the test odor was counted. Each group of flies was tested three times using water, vinegar, and isoamylacetate respectively for a total of nine tests per group of adult flies. A naïve group of 50 adult flies that had not been conditioned as larvae were also tested using the same methods. At the conclusion of the study, the average percent of adult flies from each generation of fast and slow learners, and the naïve group of flies that moved towards the conditioned odor were compared. A t-test was also conducted to determine the significance of the results.

Results

Two graphs were generated, one for selectively bred fast learners (Figure 1) and one for selectively bred slow learners (Figure 2), showing the percentage of larvae in each of four learning categories (fast, medium, slow and non-learners) across five generations of selective breeding. The P1 generation is the same group of flies shown in both graphs since these are the flies conditioned and tested at the initiation of the experiment. In the P1 generation, the number of fast and non learners was approximately equal. For selectively bred fast learners (Figure 1) the number of fast learners increased over the generations, while the number of non-learners decreased over the generations. By the F4 generation, the non-learners were bred out. The number of medium and slow learners remained approximately the same across the generations. In the

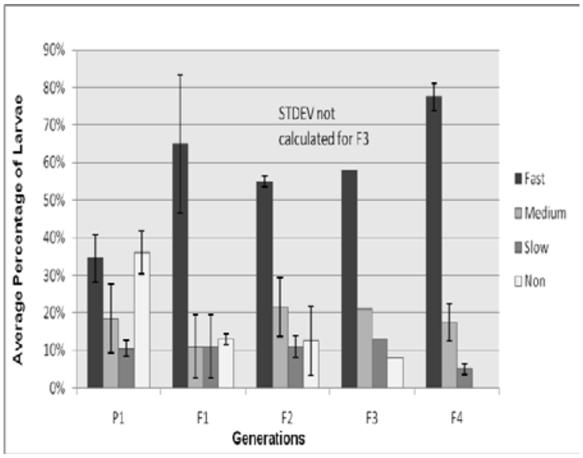


Figure 1. Selectively Bred Fast Learners : Percentage of Flies in Four Learning categories Across Four Generations. The graph depicts the average percentage of larvae selectively bred as fast learners that were designated as fast, medium, slow, and non-learners through the P1-F4 generations

graph showing results for the selectively bred slow learners (Figure 2) the number of fast learners increased across the generations, while the number of non-learners decreased across the generations. The number of medium and slow learners remained approximately the same across the generations. T-test results indicate a significant difference between the percentage of fast learners and all other learning categories in the selectively bred fast group in generations F1-F4 as well as a significant difference

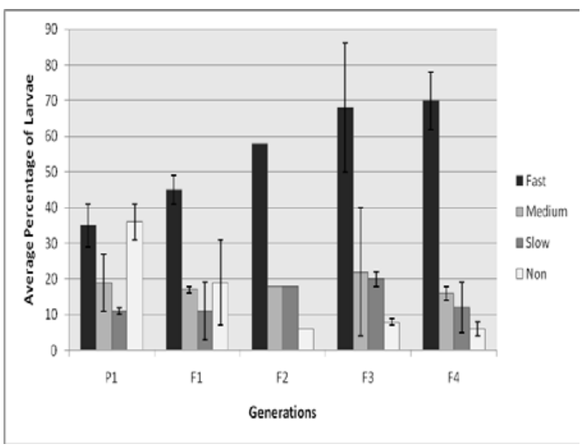
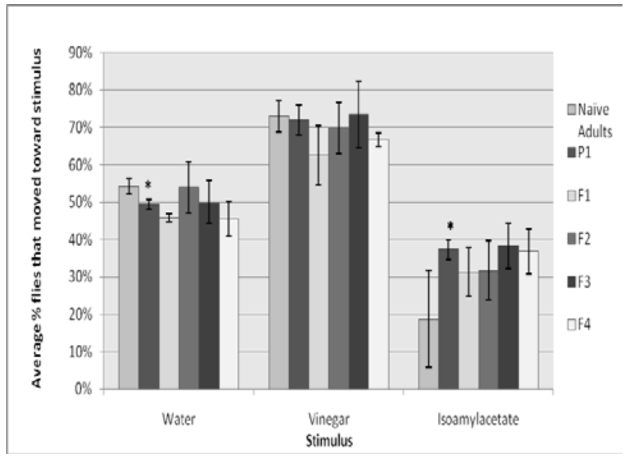


Figure 2. Selectively Bred Slow Learners: Percentage of Flies in Four Learning Catagories Across Four Generations. The graph depicts the average percentage of larvae selectively bred as slow learners that were designated as fast, medium, slow, and non-learners through the P1-F4 generations.

between the percentage of fast and both medium and slow learners in the selectively bred slow group in generations F1-F4 (p>0.05). T-tests also confirmed a significant increase in the percentage of fast learners between the P1 and F1 generations of fast bred larvae and a significant increase in the percentage of fast learners between the F1 and F2 generations of slow bred larvae (p>0.05). Differences between the percentage of fast learners in the



F1-F4 generations of fast bred larvae, between the percentage of fast learners in the P1 and F1 of slow bred

Figure 3. Selectively Bred Fast Learners: Stimulus Choice in Adult *D.melanogaster*. The graph depicts the average percentage of adult fruit flies (from selectively bred fast learning larvae and naïve adults) that gravitated towards each stimulus for the P1-F4 generations. * Indicates significance

larvae and between the percentages of fast learners in the F2-F4 generations of slow bred larvae were not significant. Two graphs were generated, one for selectively bred fast learners (Figure 3) and one for selectively bred slow learners (Figure 4), showing the results of the adult *D. melanogaster* study with the average percentage of adult flies in each generation that gravitated towards the three stimuli (water, vinegar and isoamylacetate) indicated. In

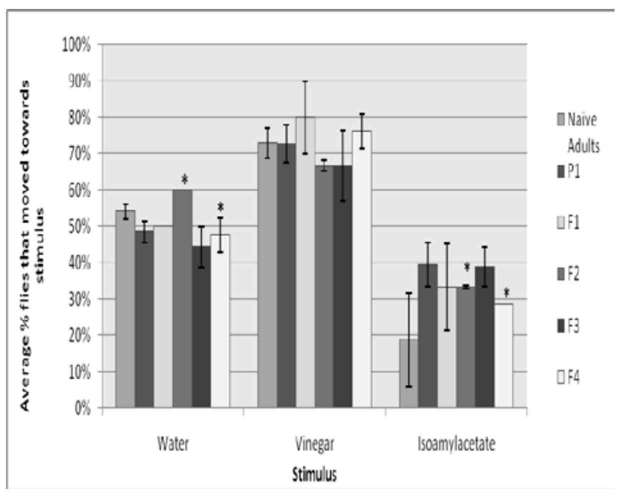


Figure 4. Selectively Bred Slow Learners: Stimulus Choice in Adult *D.melanogaster*. The graph depicts the average percentage of adult fruit flies (from selectively bred slow learning larvae and naïve adults) that gravitated towards each stimulus for the P1-F4 generations. * Indicates significance

both figures, there were no significant differences between the generations for response to each individual stimulus. In all of the flies tested there was a higher percentage of attraction for vinegar (~70%) which was expected since it was used as a positive control. Also in all the flies there was no preference towards or away from water (50% movement towards stimulus) which was also expected since water was used as a negative control. In the naïve adults there was a significant difference between movement towards water and isoamylacetate. Only 18% of these flies moved towards isoamylacetate which indicates repulsion when compared to water. However, there was a lot of variation for the naïve adults' attraction to isoamylacetate.

When comparing the naïve adults to the conditioned flies, there was an observed increase in the percentage of conditioned flies that moved toward the isoamylacetate in both figures. However, the t-test results indicated that there were no significant differences between the naïve and conditioned flies ($p>0.05$). Looking at the selectively bred fast learners (Figure 3) there is a significant difference between response to water and isoamylacetate in the P1 generation, which indicates that this generation was repelled by isoamylacetate ($t=4.0249$; $df=4$; $p<0.05$). This difference disappears however in the F1 and subsequent generations indicating less repulsion. Looking at the selectively bred slow learners (Figure 4) there was a significant difference between response to water and isoamylacetate in the F2 ($t=8.000$; $df=4$; $p<0.05$) and F4 generation ($t=4.000$; $df=4$; $p<0.05$) as well, which indicated that these generations continued to be repelled by isoamylacetate.

Conclusion

Several conclusions can be made regarding the number of conditioning experiences needed to establish associative learning in *Drosophila* larvae and the role heredity plays in this. Without even considering heredity, it can be concluded that if learning is going to occur at all it will mostly likely occur after one conditioning experience because in every generation there were more fast learners than medium or slow learners. When selectively breeding and testing fast learners it was observed that the number of fast learners is significantly higher after just one generation of selective breeding leading one to conclude that being a fast learner is hereditary. However, when selectively breeding and testing slow learners, we also observed that the number of fast learners increased significantly by the F2 generation. Perhaps selecting for the ability to associate an odor with a taste stimulus in general is enough to result in an observed increase in the percentage of fast learners while selectively breeding fast learners assures a significant increase after one generation.

The percentage of medium and slow learners did not change in either of the selectively bred colonies even when we were selectively breeding for the slow learning trait. Based on this we concluded that being a slow learner is not hereditary but may reflect the immediate physical condition or handling of the fly being tested.

By looking at the drop in the number of non-learners with each generation of selectively bred fast and slow learners we might conclude that being a learner in general (whether fast, medium, or slow) is hereditary. This result was dramatic since by the F4 generation of fast learners there are no non-learners observed; they have effectively been bred out. This was not observed in the selectively bred slow learners, although they did decrease in number over the generations. Since the actual condition of the slow learners is not known it is difficult to account for this difference. Selectively breeding fast learners does seem to result in more robust changes in population makeup. In an unpublished experiment testing and selectively breeding non-learners for 4 generations under the same conditions, all subsequent generations resembled the P1 generation in percentages of fast, slow, medium and non-learners. This suggests that being a non-learner may involve factors that can be selected against but not necessarily selected for such as poor physiological condition.

For the adult *D.melanogaster*, the data indicated that both the negative and positive controls worked for this experiment and that naïve adults seem to be naturally repelled by the concentration of isoamylacetate used in this study. Likely this concentration was too strong but this was still a useful baseline to measure against changes in the behavior of conditioned flies. Larval conditioning did seem to lower the amount of repulsion in the selectively bred fast learners but not in the selectively bred slow learners. For the selectively bred fast learners, the association made between isoamylacetate and fructose as larvae seems to persist into adulthood and be strong enough to overcome a natural aversion. Being a fast learning larvae may indicate the ability to form not just a quicker but also a stronger association between two stimuli. Alternatively, selective breeding of fast learners may have also bred for a lower sensitivity or aversion for isoamylacetate. In the future adult male and female flies should be separated and tested separately. It is expected that the observed change in behavior (loss of natural repulsion) will be even greater in the female flies since it is the female which would benefit most from using a larval association between good food and an odor to find a good place to lay eggs.

Works Cited

1. Dukas R. Ecological relevance of associative learning in fruit fly larvae. *Behavior Ecology Sociobiology*, 1998, **19**, 195-200.

2. Heimbeck G., Bugnon V., Gendre N., Haberlin C., Richard S.F. Smell and taste perception in *Drosophila melanogaster* larva: toxin expression studies in chemosensory neurons. *The Journal of Neuroscience*, 1999, **19(15)**, 6599-6609.

3. Kreher S.A., Kwon J.Y., Carlson J.R. The molecular basis of odor coding in Drosophila Larva. *Neuron*, 2005, **46**,445-456.

4. Neuser K., Husse J., Stock P., Gerber B. Appetitive olfactory learning in Drosophila larvae: Effects of repetition, reward strength, age, gender, assay type and memory span. *Animal Behaviour*, 2005, **69**, 891-8.

5. Scherer S., Stocker R.F., Gerber B. Olfactory learning in individually assayed Drosophila larvae. *Learning & Memory*, 2003, **10**, 217-225.
6. Tully T., Cambiazo V., Kruse L. 1994. Memory through metamorphosis in normal and mutant Drosophila. *The Journal of Neuroscience*, 1994, **14(1)**, 68-74.