

**A method to measure associative learning for different size larvae.**

Cooper, Richard M., and Robin L. Cooper. Dept. of Biology, University of KY, Lexington, KY. 40506-0225 USA; Email: cooper_richie@yahoo.com

Since the time of Alpatov (1929) people have been interested in studying environmental impacts on the developmental rate of larval fruit flies. Kaznowski *et al.* (1985) demonstrated that the thickness of the cuticle is gradual throughout the instar stages and does not jump with every molt cycle. This study was important since it explained why a gradual development was observed in these molting instars. This also suggests that diet could have an effect on development within an instar stage. Biochemical studies on larval fruit flies showed how the amount of proteins increases with development (Church and Robinson, 1966) and that the nitrogen and phosphate content increased with each molt stage (Watts *et al.*, 2006). These studies suggest that fruit flies are dependent on particular elements. Some factors may be developmentally rate limiting for the whole body. Despite whole larval developmental interest over the years, little attention has been given to dietary regulation on neuronal development of the larval central nervous system (CNS).

The developmental need of the CNS may be more restrictive than the whole body development due to the complexity of factors in formation and maintenance the central nervous system. There are many synapses for the refined communication. So even though whole body size might appear to be normal, one cannot assume the neural functions are fully intact. One approach to examine the effects of diet on proper development of neuronal circuits would be particular behavioral assays. It is apparent that most of the attention given to *Drosophila* behaviors has concentrated on adults, but there are a number of larval behavioral assays established. Eating and locomotion are commonly used larval behaviors (Sewell *et al.* 1975; Neckameyer, 1996; Li *et al.*, 2001). In the last few years, revolutionary studies have shown that larvae have the ability to demonstrate associative learning (Scherer *et al.*, 2003; Gerber *et al.*, 2004; Hendel *et al.*, 2005). However, the assays used are very dependent on the experimental design and might not be best suited for larvae of all developmental stages or sizes that could be altered from developmental retardation.

More direct effects on a neural circuit can also be examined. Recently, neuromodulators, such as serotonin (5-HT) and dopamine, have been shown to have a role on activity of a sensory-CNS-motor circuit in *Drosophila* larvae (Dasari and Cooper, 2004; Dasari *et al.*, 2007) as well as direct effects on the larval heart (Dasari and Cooper, 2006). So one might assume a diet restrictive in essential amino acids that are precursors to 5-HT and dopamine might impact the development in the wiring of the CNS.

In our current studies we used a simple restrictive diet of glucose and water (1 gram for 10 mls of water) and placed newly hatched embryos (1st instar) in this solution and maintained them at room temperature (21°C). With this diet larvae develop very slowly compared to ones fed a standard cornmeal-agar-dextrose-yeast medium diet commonly used for culturing. The larvae would remain within each instar for a longer time and some would take 14 days until beginning to form a pupa. The size of the larvae at each stage is drastically reduced compared to an equivalent instar stage in the controls fed an enriched diet. We determined instar stage based on the numbers of teeth present on the mouth hooks (Strasburger, 1932; Demerec, 1994).

To examine the possibility that larvae fed this restrictive diet are impaired in neuronal function we used the relatively recently described associative learning assays. We used a gustatory-visual relationship (Gerber *et al.*, 2004; Hendel *et al.*, 2005). We found that originally described procedures of having the agar dish divided into alternating quarters of light and dark for the initial and final testing for associative learning was biased toward larger larvae since they would cross the boundaries of the dark/light edges more often than smaller larvae over a given time period. If the larvae are in the middle of the dark or light pie shaped quarter then it is a relatively long way to reach a boarder transition (Figure 1A). In order to standardize the experimental design for various size larvae we used a stripped pattern (Figure 1B) and varied the strip distances as well as stripe thickness to equal two body lengths. The restrictive diet never allowed the larvae to reach the full body length of control larvae. However, each experimental group was consistent for body length within a group; we only needed to use a few sets of grids to carry out the testing on developmental time points. We feel that the new experimental design provides more opportunities for the larvae to choose their side of preference before and after associate conditioning.

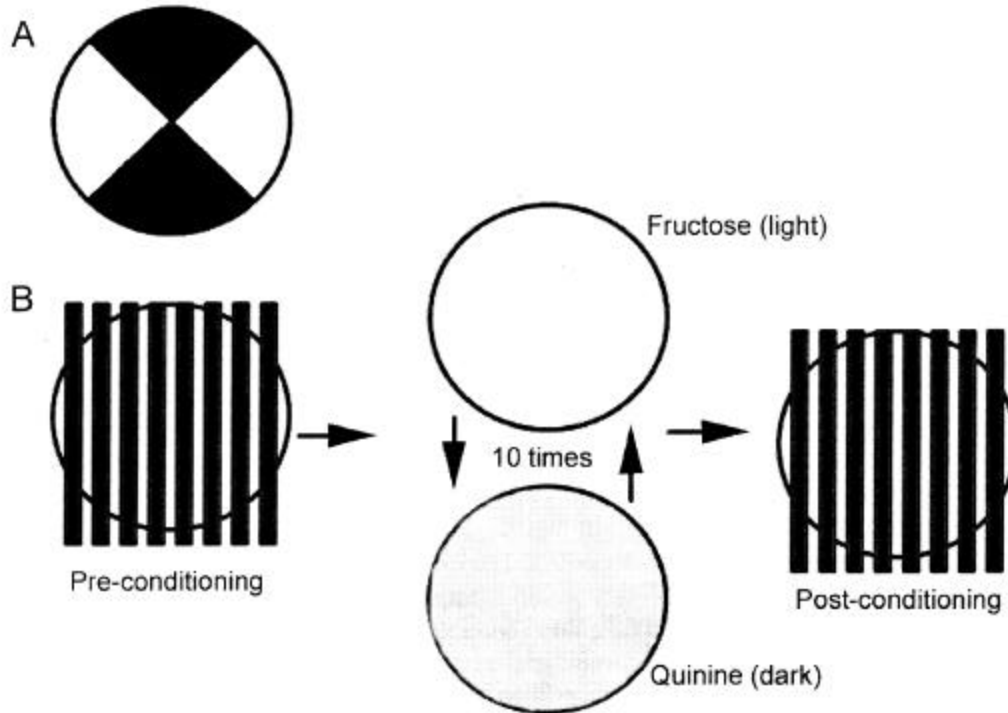


Figure 1.

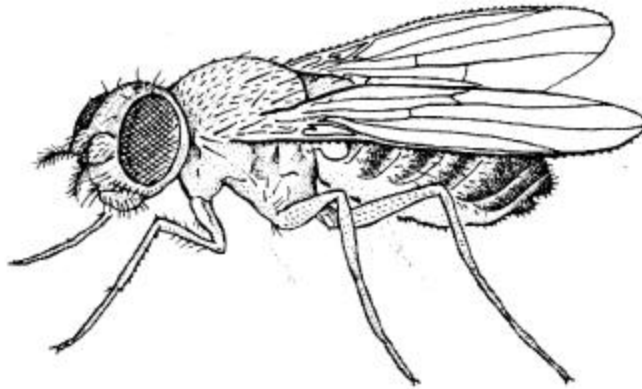
The 1% agarose plates were used for the initial and final visual (*i.e.*, light/dark) preference test. For the learning assay, two other dishes were used: one for the positive gustatory reinforcement dishes with 1% agarose and 1M fructose (FRU, purity: 99%); and one for the negative reinforcement with 1% agarose and 0.2% quinine hemisulfate (QUI, purity 93%). The gustatory reinforcers were added after the agarose was dissolved and slightly cooled just prior to pouring the agar for the plates. Glass Petri dishes were used as the agarose plates in these studies (90-mm diameter). All experiments were done in a dark room at room temperature (21°C). The dishes were placed on a light box that only allowed light to go through the grids of the dishes. The learning regime consisted of 1 min in the light with a positive gustatory reinforcement, followed by 1 min in the dark with a

negative gustatory reinforcement. This procedure was repeated 10 times. The learning takes longer than 20 min since the 1 min conditioning does not include the time for transferring the larvae. Transferring the larvae occurred as rapidly as possible (~10 seconds). For the visual preference test, the larvae are recorded every 30 sec as being either on a dark side or on a light side for a total of 5 min. The % of larvae before and after training on the various dark/light locations are then compared for significant difference in learning.

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References: Alpatov, W., 1929, *J. Exp. Zool.* 52: 407-437; Church, B., and F.W. Robertson 1966, *J. Exp. Zool.* 162: 337-352; Dasari, S., and R.L. Cooper 2004, *Neurosci. Res.* 48: 221-227; Dasari, S., and R.L. Cooper 2006, *J. Comp. Physiol. B* 176: 349-357; Dasari, S., K. Viele, A.C. Turner, and R.L. Cooper 2007, *European J. Neurosci.* 26: 424-438; Demerec, M., 1994, *Biology of Drosophila*, CSHL Press, p. 285; Gerber, B., S. Scherer, K. Neuser, B. Michels, T. Hendel, R.F. Stocker, and M. Heisenberg 2004, *J. Exp. Biol.* 207: 179-188; Hendel, T., B. Michels, K. Neuser, A. Schipanski, K. Kaun, M.B. Sokolowski, F. Marohn, R. Michel, M. Heisenberg, and B. Gerber 2005, *J. Comp. Physiol. A.* 191: 265-279; Kaznowski, E.C., H.A. Schneiderman, and J.P. Bryant 1985, *J. Insect Physiol.* 31: 801-813; Li, H., D. Harrison, G. Jones, D. Jones, and R.L. Cooper 2001, *J. Neurophysiol.* 85: 98-104; Neckameyer, W.S., 1996, *Dev. Biol.* 176: 209-219; Sewell, D., B. Burnet, and K. Connolly 1975, *Genet Res. Camb.* 24: 163-173; Scherer, S., R.F. Stocker, and B. Gerber 2003, *Learn Mem.* 10: 217-225; Strasburger, M., 1932, *Meig. Zeit. wiss. Zool.* 140: 539-649; Watts, T., A. Woods, S. Hargand, J. Elser, and T. Markow 2006, *J. Insect Physiol.* 52: 187-193.

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Prepared at the
Department of Zoology
University of Oklahoma
Norman, OK 73019 U.S.A.