

ORIGINAL ARTICLE

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Ecological relevance of associative learning in fruit fly larvae

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Abstract A few invertebrate models have been used for studying neurobiological and molecular aspects of associative learning. The ecological and evolutionary aspects of associative learning in these invertebrates are not yet well understood. To further this knowledge, I tested fruit fly larvae for their ability to learn to associate odors with preferred environmental conditions. The larvae learned to avoid odors associated with predation and to prefer odors associated with high-quality food, but failed to learn about odors associated with optimal temperature. It appears that the larvae possess a general ability to evaluate a suite of environmental parameters and associate preferred conditions with relevant stimuli.

Key words Fruit fly · Associative learning · Invertebrate learning

Introduction

The central role of primitive nervous systems is intercellular communication and coordination. More developed nervous systems show an increased capacity to acquire and store an individual's experience, which is learning and memory. Most notably, associative learning allows an individual to acquire a neural representa-

tion of a new association between a stimulus and an environmental state that may affect fitness. The molecular biology of learning in general and associative learning in particular has been subjected to intense research in recent years (Selverston 1985; Alkon and Woody 1986; Anderson 1989; Beer et al. 1993; Glanzman 1995; Baily et al. 1996). A key animal model for such research is the fruit fly, *Drosophila melanogaster* (reviewed by Davis 1996; Tully 1996). Well-controlled experiments have documented associative learning in both adults and larvae of the fruit fly (Quinn et al. 1974; Aceves-Pina and Quinn 1979; Tempel et al. 1983; Tully and Quinn 1985). However, the function and adaptive significance of associative learning for fruit flies remains largely unexplored. Further study of this issue may help us understand the ecological circumstances that favor the evolution of associative learning.

Most flies of the genus *Drosophila* feed on yeasts and bacteria associated with decaying plant and fungal material. A fruit fly larva developing on a substrate such as decaying fruit may encounter a wide variety of plant compounds, yeast and bacterial species, and biochemical and physical environments. This array of biotic and abiotic factors shows a high spatiotemporal variation (Parsons and Stanley 1981; Begon 1982; Shorrocks 1982). Larval growth is probably feasible without associative learning: all that larvae have to do is detect and consume their favored yeasts and bacteria under preferred physiological and chemical conditions. But larvae may increase their growth rate or survival probability if they learn to associate certain stimuli with favorable conditions and then pursue these stimuli throughout development. If so, then larvae should be able to recognize some key ecological conditions and learn to identify these factors through associated stimuli. I tested this prediction with *D. melanogaster* larvae. Specifically, I asked whether larvae would learn to associate distinct odors with (1) rich versus poor food sources, (2) safe versus unsafe microhabitats, and (3) an area at optimal warm temperature versus a cold region.

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Methods

The flies

D. melanogaster flies of the Canton-S strain were obtained from the laboratory of M. Sokolowski at York University, Canada. This strain showed the best learning scores among several strains tested by Tully and Quinn (1985). A population of a few thousands flies was maintained in 80-ml glass jars containing food medium at 24 ± 2 °C in a 12:12 h light/dark cycle with lights on at 7:00 a.m. One liter of the food medium contained 75 g corn meal, 32 g brewer's yeast, 60 g glucose, 30 g sucrose, 9 g sodium potassium tartrate, 7 g calcium chloride, 20 g agar powder, 2 g methylparaben, and distilled water.

Every morning, I placed about 100 mature flies in a glass container with fresh medium. The flies were removed about 7 h later, when the medium contained a few hundred eggs. Third-instar larvae from that container were used approximately 5 days later. To remove the larvae from the container, its content (medium and larvae) were placed in a cup containing 15% sucrose water and the larvae were allowed to float to the surface; they were then placed on a mesh screen and washed with distilled water. Using a moist thin artist's paint brush, I placed approximately 175 larvae inside each of four small petri dishes; each dish represented one replicate. With the exception of the food experiment (see below), all dishes contained a thin disk of food medium consisting of 5% brewer's yeast, 5% glucose, 4% agar and 2% methylparaben (w/v).

General experimental protocol

I conducted three experiments to test the ability of larvae to learn to associate odors with food types, predation risk, and temperature. In each experiment, there was an unambiguously preferred treatment (the "good" treatment) that larvae were expected to learn to associate with and distinguish from the other "bad" treatment based on the associated odors. The treatments employed are detailed below for each experiment. Larval behavior indicated that they indeed perceived the bad and good treatments as such: fewer larvae were engaged in feeding during the bad treatment and more attempted to leave the petri dish.

The two odorants used in all three experiments were ethyl acetate (EA) and isoamyl acetate (IA). These organic compounds commonly occur in plants and animals. Larvae show strong attraction to these odorants (Rodrigues 1980), which were successfully used in associative learning experiments with electric shock as

the unconditioned stimulus (Tully et al. 1994). In a preliminary choice experiment, experimentally naive larvae expressed no preference for either odorant: $53 \pm 0.05\%$ (mean \pm SE) of the larvae chose EA over IA (Wilcoxon signed-rank test, $P > 0.1$, $n = 8$ replicates with 100 larvae per replicate).

Each of the three experiments consisted of the same three general conditions, alternating sessions of two types, and replicates of each condition included all possible combinations of odorant and treatment (Table 1). In the odors and treatments condition (Table 1, condition A), larvae were first placed in a dish subjected to one of two treatments and containing a 5- μ l solution of one of two odorants inside a microcapillary tube. After the first session, the larvae were transferred to the second dish, which was subjected to the other treatment and contained 5 μ l of the other odorant. When the second session ended, I transferred the larvae back to the first dish, where they experienced the first treatment and a 5- μ l microcapillary tube refilled with the first odorant; at the end of that session, the larvae experienced again the other session type, and so on. This sequence of alternating sessions of two types was repeated for a total of 12 30-min sessions in experiment 1, and 6 60-min sessions in experiments 2 and 3. That is, the larvae spent about 6 h in training; during half that time, they experienced one treatment associated with one odorant, and during the other half, they were exposed to the other treatment and other odorant.

The second and third conditions were controls designed to reveal whether significant results indicating associative learning in the odors and treatments condition can be obtained in conditions where odors and treatments are not paired. Hence, the second condition had odors only, and the third condition had treatments only. In the odors-only condition (Table 1, condition B), larvae spent the first session inside a small petri dish with a disk of yeast medium and a 5- μ l microcapillary tube containing one odorant; then they were transferred to another dish containing a disk of yeast medium and a 5- μ l microcapillary tube containing the other odorant. The two dishes offered an identical environment to the larvae except that each had a distinct odor. As before, this two-session sequence was repeated six times in experiment 1 and three times in experiments 2 and 3 (Table 1, condition B).

In the treatments-only condition (Table 1, condition C), larvae spent the first session inside a small petri dish subjected to one treatment; they were then transferred to another dish and experienced the other treatment. The two dishes offered identical "natural" odors to the larvae. This two-session sequence was repeated six times in experiment 1 and three times in experiments 2 and 3 (Table 1, condition C).

Immediately after the end of each training period, the larvae were tested for their odor preferences. I placed the larvae at the center of a 90-mm petri dish containing 5% agar medium. At op-

Table 1 General protocol used in the three experiments. Each experiment consisted of three conditions (A, B, and C), two session types (1 and 2) and four or two replicate types (*Roman numerals*) consisting of all possible combinations of odor and/or treatment; larvae within the same replicate type were transferred several times

Replicate type	Session type 1		Session type 2	
	Odor	Treatment	Odor	Treatment
Condition A: odors and treatments				
I	IA	Good	EA	Bad
II	IA	Bad	EA	Good
III	EA	Bad	IA	Good
IV	EA	Good	IA	Bad
Condition B: odors only				
I	IA	–	EA	–
II	EA	–	IA	–
Condition C: treatments only				
I	–	Good	–	Bad
II	–	Bad	–	Good

between their particular session type 1 and session type 2 before the test. In each experiment, one treatment (*Good*) was obviously preferable over the other treatment (*Bad*). See Methods for details (EA ethyl acetate, IA isoamyl acetate)

posite sides of the dish, I placed a 5- μ l microcapillary tube containing EA at one side and IA at the other. The positions of odorants were switched between replicates. In experiments 2 and 3, I also placed disks of yeast medium 1 cm in diameter near each capillary tube. This modification was in response to observations in experiment 1 of larvae arriving near a capillary tube and then crossing to the other, apparently in search of food. Larvae moved quickly towards the odor gradients (see also Aceves-Pina and Quinn 1979). Five minutes after introducing the larvae, I counted their numbers in each half of the dish while ignoring the ones (always only a few) within 1 cm of the center.

To calculate the learning scores in the odors and treatments condition, I averaged the percentages of larvae choosing the odor associated with the good treatment in two replicates, one where EA was associated with the good treatment, and the other where IA was associated with the good treatment. This way, odor biases are canceled out and, consequently, a random choice of odor would result in a 50% score, while perfect learning would result in a 100% score. For the control conditions, where there is no correct choice, I randomly assigned, before the experiment, "expected" preferences, half the time to EA and half to IA. I then calculated test scores by averaging the percentages of larvae choosing the assigned odor in two replicates, one where EA and the other where IA was the assigned odor. Here, again, a 50% score would indicate random choice. A score significantly higher than 50% would suggest that another factor besides associative learning biases test outcomes.

Note that each learning score representing a single data point was based on the choice of 200–300 larvae. First, out of the 175 larvae per petri dish, 100–150 survived to the test (most of the mortality was caused by larvae leaving the petri dish and dehydrating); second, each learning score is the average from two reciprocal replicates. Learning is an individual- rather than a population-level phenomenon. Hence, it is usually appropriate to evaluate learning by each individual separately. However, research on associative learning in *Drosophila* and a few other invertebrate models suggests that reliable learning scores can usually be obtained only through using populations because of large individual variation in behavior. Control experiments suggest that learning scores of individuals tested independently are similar to scores obtained from populations (Quinn et al. 1974; Tempel et al. 1983).

Overall, each of the three experiments had 16 replicates of the odors and treatment condition, and 16 replicates for the odors-only and treatments-only conditions. The following three sections describe details of the general protocol for each of the three experiments.

Experiment 1: learning about food types

The two treatments were high- and low-quality food media. The high-quality food contained 10% brewer's yeast, 10% glucose, 2% agar 0.2% methylparaben (w/v), and distilled water. The low-quality food consisted of 1% quinine sulfate, 2% agar, 0.2% methylparaben, and distilled water. Thus, the high-quality food provided larvae with ample protein and carbohydrates while the low-quality food was bitter and lacked protein and carbohydrates.

After the completion of the three experiments, I conducted a follow-up experiment in an attempt to improve the learning scores achieved by larvae with the original protocol. In the eight replicates of the new experiments, a session duration was increased from 30 to 45 min, a two-session sequence was repeated four times, the high-quality food was as before, but the low-quality food had 1% brewer's yeast instead of quinine. These slight modifications were based on my season-long experience with larval behavior.

Experiment 2: learning about predation risk

The two treatments were simulated predation and no predation. During the simulated predation treatment, every 10 min, the larvae

were gently carried to the edge of the food medium with a fine brush and a single larva was crushed with the wooden tip of the brush. This treatment simulates disturbance at a food medium such as fruit, caused by movements of a large insect or a small mammal or bird searching for food. While the disturbance was experienced by all larvae, I do not know to what extent larvae could notice the larval death. In the no-predation treatment, larvae were allowed to feed without disturbance.

Experiment 3: learning about temperatures

Here, the good treatment was a temperature of 24 ± 2 °C. This temperature is within the 5 ° range at which larvae show the highest combination of growth rate and survivorship. The bad treatment was a temperature of 14 ± 2 °C, well within normal temperatures experienced by larvae, but larval growth is much slower (Ashburner 1989). The cold temperature was created by placing the larvae in a Styrofoam box containing ice.

Results

Experiment 1: learning about food types

In the odors and treatments condition, $64 \pm 3\%$ (mean \pm SE) of the larvae chose the odor that had been associated with the high-quality food during training (Wilcoxon signed-rank test, $P < 0.02$, $n = 8$; Fig. 1a). Of the eight learning scores, seven had values above the random 50%, with a range of 47–80%. By contrast, larval choices were random in the two control conditions ($P > 0.1$, $n = 8$; Fig. 1a). This indicates that the outcome of the odors and treatments condition reflects true associative learning. Results of the follow-up experiments were very similar to those in the original experiments; here the average learning score was $66 \pm 3\%$ ($n = 4$).

Experiment 2: learning about predation risk

In the odors and treatments condition, $73\% \pm 4$ of the larvae chose the odor that had not been associated with simulated predation during training ($P < 0.01$, $n = 8$; Fig. 1b). All eight learning scores had values above the random 50%, with a range of 51–88%. By contrast, larval choices were random in the two control conditions ($P > 0.1$, $n = 8$; Fig. 1b).

Experiment 3: learning about temperatures

In the odors and treatments condition, $56\% \pm 5$ of the larvae chose the odor that had been associated with the warm temperature (24 °C) during training. This is not significantly different from 50% ($P > 0.1$, $n = 8$; Fig. 1c). Learning scores varied between 42–79%. By contrast, larval choices were slightly above random in the two control conditions ($P < 0.05$, $n = 8$; Fig. 1c).

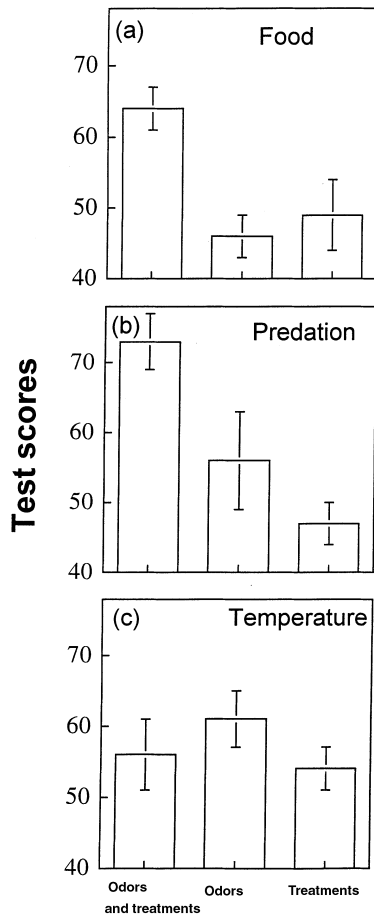


Fig. 1 Average scores (\pm SE) of larvae tested after training under the conditions of paired odors and treatments (which allow associative learning), and the two controls of odors alone and treatments alone. A score of 50% indicates no learning, and a 100% score is perfect learning. Results are for learning about food types (a), predation risk (b), and temperature (c)

Discussion

Fruit fly larvae learned to associate odors with predation risk and food types but not with temperature. These results add ecological relevance to the only two previous studies on associative learning in fruit fly larvae, which used electric shock as the unconditioned stimulus (Aceves-Pina and Quinn 1979; Tully et al. 1994). It is premature to discuss the meaning of the relatively high learning scores for predation, low scores for food, and lack of associative learning about temperature before further experimental data are available. It is feasible, however, that the larvae do not attend to odor cues associated with temperature because they have little ability to alter body temperature through movement. It is also possible that the larvae learn about, or respond more strongly to, cues about predation given the larger effect this may have on fitness compared to food quality.

It might be argued that the learning scores reported here are rather low and unconvincing. The statistically

significant scores, however, demonstrate that the larvae can learn the appropriate responses of seeking higher-quality food and escaping predation. The goal of this study has been to understand the evolution and potential adaptive significance of associative learning in a species with limited learning capacity. This is analogous to studying the evolution of flight or vision through the examination of species depicting what appears to be the ancestral condition. Because the choice of study species is based on the expectation that it depicts only a primitive version of the fully developed trait, experimental results showing that this is indeed the case are to be expected.

It is difficult to determine whether the experimental protocol hindered or enhanced larval learning compared to their potential learning ability in nature. For example, on the one hand, the continuous transfer of larvae between dishes may have caused severe disturbance that hindered learning; on the other hand, the use of only two distinct stimuli and two extreme environmental states is rather artificial and could enhance learning compared to learning under noisy natural settings.

The fact that fruit fly larvae can learn about ecologically relevant parameters suggests that such learning can increase larval fitness in natural settings. That is, under certain environmental conditions such as the availability of rich food patches with a distinct odor within a single fruit, learning to seek that odor can decrease larval developmental time and hence increase fitness. Similarly, learning to associate an odor with disturbance or predation may help larvae to initiate a timely escape that can increase survival rate. To substantiate this connection between learning and fitness, one must demonstrate that learning about specific food or predators does indeed increase fitness compared to the alternatives of seeking any potential food and escaping disturbance when it is noticed directly.

At the proximate level, at least four requirements must be met for an animal to show associative learning. These are (1) an ability to distinguish between relevant sensory stimuli, (2) a capacity to identify the optimal environmental state among available alternatives, (3) a machinery for recording information, and (4) a mechanical ability to move towards or away from certain stimuli. All four conditions exist in all arthropods that have been closely studied (see Corning et al. 1972; Marler and Terrace 1984; Papaj and Lewis 1993). Moreover, extensive research on the molecular biology of learning and memory suggests that associative learning is a basic neuronal process rather than a mechanism requiring some complex features of neural networks (Kandel et al. 1995; Baily et al. 1996).

Given that the proximate preconditions for associative learning are met in most invertebrates, we can turn to an ultimate analysis of what an organism such as a fly larva *should* learn. This analysis is in general agreement with previous discussions on the evolution of learning (e.g., Mayr 1974; Johnston 1982; Gould 1986; Papaj and Prokopy 1989; Stephens 1991; Dukas 1998a). Learning

can incur costs, mostly in terms of time and energy spent exploring alternatives; hence there is no need to learn about associations that do not change often over evolutionary time. So, for example, the fly larvae can depict innate attraction to the smell of yeast or any odor associated with decomposition, indicating the occurrence of yeast or bacteria. This assertion is in agreement with Rodrigues' (1980) data, which showed that fruit fly larvae were attracted to all chemicals tested. The larvae can also show an innate preference for a certain suite of abiotic parameters such as temperature, humidity, and light intensity. However, there are many additional stimuli that can indicate the presence of some environmental events affecting fitness. If the number of such stimuli is very large, and if each occurs relatively rarely during a larval lifetime, there would be weak selection on acquiring innate responses to such stimuli. A more likely alternative is to learn to associate a stimulus with the environmental event with which it is associated. Such associative learning can increase fitness if the association between the stimulus and the environmental event does not change too quickly in relation to the time it takes to learn about that association.

There may be fundamental differences between two categories of learning: programmed learning and incidental learning. Programmed learning is the category typically discussed by students of learning. It means that an animal possesses a strong predisposition to learn about something, be it kin phenotype, song, local environmental features, or site, smell, and color of flowers (see for examples, various contributors in Marler and Terrace 1984; Papaj and Lewis 1993; Dukas 1998b). By contrast, incidental learning implies that an animal has a general ability to evaluate a suite of biotic and abiotic conditions and various environmental stimuli; when a certain favorable or harmful condition is repeatedly associated with a specific stimulus, the individual may notice that association and learn to seek or avoid that stimulus. Programmed learning allows an individual to attend to a specific, well-defined set of stimuli and thus learn rapidly; in contrast, incidental learning implies an unfocused search for associations, which may result in slow and more variable learning by different individuals. In other words, programmed learning involves an active search for information such as sampling alternative flower species or active exploration of the environment for landmarks. Incidental learning, on the other hand, means depicting innate behavioral preferences but supplementing these with knowledge acquired over time about associations between some stimuli and environmental events. The capacity of fly larvae to learn about food and predation can be categorized as incidental learning. Incidental learning may provide the link between non-associative learning (i.e., habituation and sensitization) and programmed learning.

In the past two decades, behavioral experiments on whole animals and cellular and molecular studies have fully transformed the study of animal learning. It is now widely appreciated that even tiny, short-lived animals

are capable of associative learning (e.g., Marler and Terrace 1984; Papaj and Lewis 1993; Dukas 1998b), and that identical molecular mechanisms of learning are shared by invertebrates and mammals (reviewed by Kandel et al. 1995; Baily et al. 1996). It is now timely to advance the study of animal learning by carefully studying one or a few invertebrate models for the interplay between their ecologies, evolution, phylogeny, and specific learning capacities (see Wright et al. 1996 for a recent example). The fruit fly is an ideal candidate for such long-term systematic study because of the enormous knowledge on its genetics, ecology, and evolution (e.g., Ashburner et al. 1976; Ashburner 1989). Moreover, a unique property of the fruit fly system is the existence of a few well-studied learning-deficient mutants (Tully 1996) that can be employed for evaluating evolutionary and ecological aspects of learning.

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