



Appetitive olfactory learning in *Drosophila* larvae: effects of repetition, reward strength, age, gender, assay type and memory span

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Associative plasticity is a basic characteristic of behaviour. We analysed associative olfactory learning in larval *Drosophila*, using a reciprocal conditioning assay. One group of animals was rewarded with fructose in the presence of odour A but not in the presence of odour B (A+/B); the companion group received reciprocal training (A/B+). During the test, larvae were given a choice between A and B; those that had received A+/B training showed a higher preference for A than reciprocally trained larvae did. As all other parameters were equal between groups, this difference was exclusively due to associative learning. Learning reached an asymptote after as few as three training trials and 2.0 mol/litre of fructose yielded asymptotic learning. Learning was not modulated by larval age tested 4, 5 and 6 days after egg laying or by gender. En masse assays confirmed the lack of gender differences. Memory was fully stable for at least 30 min. These experiments provide a basis for future investigations into the cellular and molecular underpinnings of appetitive olfactory learning in larval *Drosophila*.

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Predictive, associative plasticity is a fundamental feature of both synaptic physiology (Bi & Poo 2001) and behaviour (Rescorla 1988). A major topic in behavioural neuroscience is therefore to understand how associative plasticity on the synaptic level relates to associative changes in behaviour (Martin et al. 2000). It is critical for such an endeavour to choose a relatively simple and experimentally accessible experimental system. The *Drosophila* larva meets these demands and is thus ideally suited for this kind of research programme. It combines the advantages of a genetically tractable model organism for behavioural research, allowing researchers to turn on and off defined, small sets of neurons using transgenic techniques (Phelps & Brand 1998; Kitamoto 2001; Sokolowski 2001; Heisenberg 2003) with a nervous system that has ten to a hundred times fewer cells than that of adult flies (e.g. Stocker 2001; Python & Stocker 2002), and ca. 10 million times fewer than that of humans. Despite this simplicity, *Drosophila* larvae can learn well, both associations between visual stimuli and gustatory reinforcement (Gerber et al. 2004) and associations between odours and gustatory reinforcement (Dukas 1999;

Scherer et al. 2003; Hendel et al., in press). Thus, the *Drosophila* larva can be used for a multilevel approach to understand associative plasticity. In this paper, we contributed to this research programme on the behavioural level, by analysing appetitive olfactory learning.

Our conditioning assay was based on a reciprocal, differential conditioning assay for olfactory learning (Scherer et al. 2003; Hendel et al., in press) and used individually assayed animals. One group of animals was rewarded in the presence of stimulus A but not in the presence of another stimulus B (A+/B) (A and B being two odours in the olfactory version of these paradigms, or light and dark in the visual version). The companion group received reciprocal training (A/B+). Thus, both groups received identical handling and exposure to all stimuli; what differed between them was exclusively the combination of stimuli with reinforcement. To test for learning, we gave larvae a choice between A and B. If associative learning does occur, larvae that had received A+/B training are predicted to show a higher preference for A than larvae that had received the reciprocal A/B+ training. Given that all other parameters are equal between groups, such a difference would be exclusively the result of associative learning.

We investigated the following questions. (1) Does learning improve with repeated training? (2) Do stronger rewards improve learning? (3) Is learning dependent on larval age? (4) Does larval learning differ between genders?

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(5) Are en masse assays feasible? (6) Can memory still be demonstrated up to 90 min after training?

METHODS

We used third-instar feeding-stage larvae, from the Canton-S wild-type strain, aged 5 days (range 96–120 h) after egg laying, unless stated otherwise. Third instars were chosen to keep results comparable with our earlier studies (Scherer et al. 2003; Hendel et al., in press). Flies were kept in mass culture and maintained at 25°C, 60–70% relative humidity and a 14:10 h light:dark cycle. Experiments were performed in red light under a fume hood at 20–24°C room temperature.

As olfactory stimuli we used 1-octanol (OCT, purity: 99.5%; Fluka/Sigma-Aldrich, Steinheim, Germany) and amyl acetate (AM, purity: 99%, diluted 1:50 in paraffin oil; both Fluka/Sigma-Aldrich). We applied odorant by adding 10 µl of odour substance to teflon containers (inner diameter 5 mm) which could be closed by a perforated lid (seven holes, 0.5 mm diameter).

The Learning Paradigm

We compared individual larvae which underwent either of two reciprocal training regimes (Fig. 1a): one received AM with appetitive reinforcement by fructose (FRU, see below) (AM+) and OCT without reinforcement (AM+/OCT); the second group was trained reciprocally (AM/OCT+). Then, larvae from both treatment conditions were individually tested in a choice situation for their preference between AM and OCT. Associative learning is indicated by differences between individuals from reciprocal treatment conditions during the test. This

conclusion is compelling as, during training, individuals from the AM+/OCT group and the AM/OCT+ group had identical exposure to odorants and reward; what differed between treatment conditions was solely the contingency between them. The reciprocal groups, as well as the groups within each parametric study, were run at the same time to avoid false positive differences between groups which could result from spurious variation in behaviour over time. In particular, the fact that reciprocally trained animals were run alternately allows stringent pairing of data for the calculation of a learning index (LI; see below).

Petri dishes (Sarstedt, Nümbrecht, Germany), 85 mm inner diameter, were filled with 1% agarose (electrophoresis grade; Roth, Karlsruhe, Germany), allowed to solidify, covered with their lids, and left untreated until the following day. As reinforcer we added 2 mol of FRU (purity: 99%, Sigma-Aldrich) to 1 litre of agarose solution, unless stated otherwise.

Immediately before the experiments, we replaced the regular lids of the petri dishes with lids perforated in the centre by 15 1-mm holes to improve aeration. A spoonful of food medium containing larvae was taken from the food bottle and transferred to a glass vial. On demand, eight larvae were taken, briefly washed in tap water and together transferred to a training plate. These plates contained either pure agarose or had FRU added to them. Experimenters were blind with respect to presence or absence of the reinforcer. This was decoded only after the experiment. For half of the cases, we started with pure agarose as the substrate, and for the other half of the cases, we started with a FRU-containing plate.

Immediately before a training trial, two containers loaded with the same odorant each (for details see below) had been placed on opposite sides of the plate, 7 mm from the edges. Then, the lid was closed and the larvae were

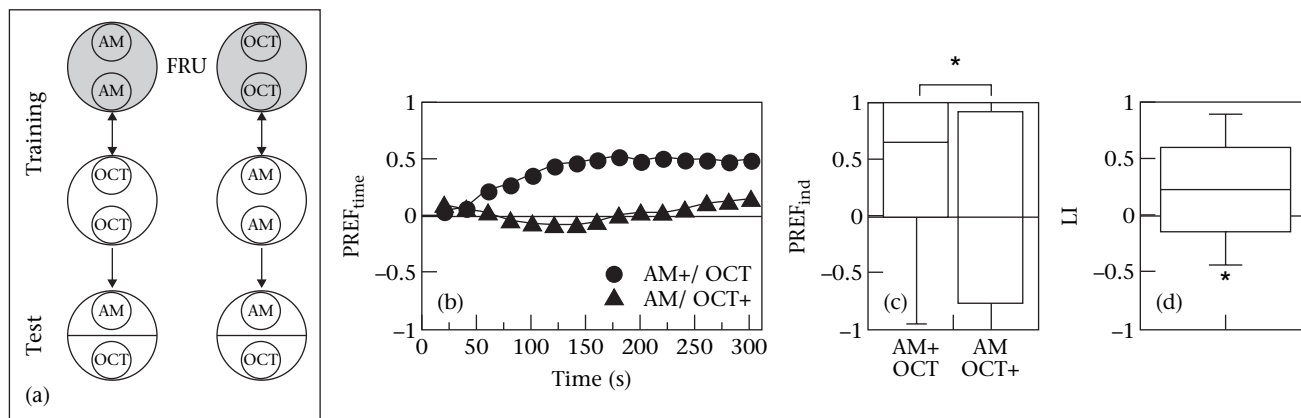


Figure 1. (a) Diagram of the procedure for the conditioning experiment; for half of the cases, the sequence of training trials within each reciprocal group was as indicated (i.e. AM+/OCT and OCT+/AM), whereas for the other half of the cases (not shown), the sequence of training trials was reversed (i.e. OCT/AM+ and AM/OCT+). AM and OCT refer to the odorants used for training (amyl acetate and 1-octanol, respectively). (b) Odour preferences for each time point of the test calculated as the number of larvae on the AM side minus the number on the OCT side, divided by the total number of larvae. Thus, positive values indicate that a majority of larvae were recorded on the AM side at that time, whereas negative values indicate that a majority were on the OCT side. (c) Preference values were calculated for each larva as the number of times a given larva was observed on the AM side during the test, minus the number of times that it was observed on the OCT side, divided by the total number of observations. Positive values indicate AM preference and negative values OCT preference. (d) Learning index (LI) calculated for pairs of larvae that underwent either of the reciprocal training regimes, e.g. either AM+/OCT or AM/OCT+, by subtracting the preference values of both larvae and dividing the result by two. * $P < 0.05$, Mann–Whitney U test (c) and one-sample sign test (d). The box plots represent the median as the middle line and 10 and 90, and 25 and 75%, quantiles as whiskers and box boundaries, respectively.

allowed to move about the plate for 1 min. Unless stated otherwise, larvae were then transferred to a completely empty petri dish for a 1-min intertrial interval (ITI). Then, they were transferred to a plate with the alternative odorant and the respective other substrate for 1 min. The number of times this cycle was repeated is stated along with the results. We used fresh assay plates for each trial.

After this training, each larva was as soon as possible (2 min; unless stated otherwise) individually tested for its odour choice; thus, larvae were trained in groups of eight, but tested as individuals. We placed each larva on a fresh, pure agarose assay plate with a container of AM on one side and one of OCT on the other side to create the desired choice situation; sides were changed for every other set of larvae. Individual larvae were placed in the centre of the petri dish, the lid was closed and the position (defined by the mouth hooks) noted every 20 s for 5 min as 'neutral' (a 7-mm-wide zone in the middle of the assay plate), 'AM', or 'OCT'. As soon as larvae moved on to the lid or the odorant containers, or dug into the agarose (<5% of animals) we stopped collecting data.

The analysis of behaviour during the test involved three steps (Fig. 1). We first calculated the odour preferences of the larvae for each time point as the number of larvae on the AM side minus the number on the OCT side, divided by the total number of larvae observed:

$$\text{PREF}_{\text{time}} = \frac{(\text{number of larvae}_{\text{AM}} - \text{number of larvae}_{\text{OCT}})}{\text{number of larvae}_{\text{TOTAL}}} \quad (1)$$

Thus, these values range from -1 to 1 . Positive values indicate that the majority of larvae were on the AM side at a given time, whereas negative values indicate their localization on the OCT side.

Second, we calculated an odour preference for each larva. We determined the number of times a given larva was on the AM side during the test minus the number of times that it was on the OCT side, divided by the total number of observations:

$$\text{PREF}_{\text{ind}} = \frac{(\text{number of observations}_{\text{AM}} - \text{number of observations}_{\text{OCT}})}{\text{number of observations}_{\text{TOTAL}}} \quad (2)$$

Thus, a positive value indicates a preference for AM of a given larva, and a negative value a preference for OCT.

Third, to determine whether these preferences depended on training regime, we took the PREF_{ind} values from those pairs of individual larvae from either training condition that had been trained and tested close in time and calculated a learning index ranging from -1 to 1 as:

$$\text{LI} = (\text{PREF}_{\text{ind AM+}/\text{OCT}} - \text{PREF}_{\text{ind AM}/\text{OCT+}}) / 2 \quad (3)$$

Accordingly, positive LI values indicate associative learning. We compared LIs against random levels with (nonparametric) one-sample sign tests and (parametric) one-sample t tests. Such t tests were possible as the distribution of LIs was never significantly different from

a normal distribution (tested by Kolmogorov–Smirnov tests; not shown); these t tests exclude possible false positive results of the one-sample sign test, which could have resulted from sampling errors when assigning larvae to pairs. That is, such sampling errors might change the distribution of LI values and hence change the median; for small sample sizes, which is not the case in this study, this could affect the outcome of nonparametric tests. As such sampling errors, however, leave the mean LI unchanged, t tests are insensitive to these potential errors. In all cases, results were concordant between the nonparametric one-sample sign test and the parametric one-sample t test (not shown). For further discussion see Hendel et al. (in press). To compare LIs between treatment conditions, we used Kruskal–Wallis tests, which, when we found a significant effect, were followed by Mann–Whitney U tests. All statistical analyses were two tailed and were performed with StatView (Abacus Concepts, Berkeley, California, U.S.A.) for the Macintosh.

To test for an effect of age, we took larvae aged 4, 5 or 6 days after egg laying. They were all stage 3 larvae, and in all cases were taken from the food medium so the 6-day-old larvae were probably still in their feeding stage.

To test for an effect of gender, we individually placed the trained larvae in small food vials to allow development into adulthood. Then, we determined gender and calculated LIs separately for each gender. We preferred this procedure to sexing larvae directly before training, because we found in pilot experiments that such sexing can be erroneous; this is because although males can be judged unambiguously by their gonad imaginal discs, there is no positive marker for females. Thus, in cases where the gonad imaginal discs were invisible, larvae may be falsely classified as females.

En Masse Assay

The en masse assay differed from the above individual-animal assay in that (1) a group of 30, rather than eight, larvae was trained; (2) testing took place for the whole group of larvae, rather than for each one separately; (3) no ITI was used. To calculate preferences, we counted the larvae on the AM side, subtracted from this value the number of larvae on the OCT side and divided this difference by the total number of larvae; no neutral zone was defined for this kind of assay. We counted larvae 3 min after the test had started, to allow the larvae to disperse. The preference values from reciprocally trained larvae were then treated as above for the calculation of LI.

To test for an effect of gender in the en masse assay, we took the larvae from the AM side and OCT sides and put them into separate food vials to allow development to adulthood. Then, we determined gender and calculated LIs separated by gender.

RESULTS

Appetitive Olfactory Learning

After eight learning trials, larvae that had received AM+ /OCT training showed a higher AM preference than

those that had received AM/OCT+ training (Mann–Whitney test: $Z = -2.74$, $N_1 = 68$, $N_2 = 75$, $P < 0.05$; Fig. 1b, c). When the preference values of only those 68 larvae that could be assigned to pairs for the subsequent calculation of LIs were compared in a paired fashion with a Wilcoxon signed-ranks test, we also found that AM preferences were higher after AM+/OCT than after AM/OCT+ training ($Z = -3.06$, $N = 68$, $P < 0.05$). This difference can be quantified by a median LI of 0.2, reflecting that LIs were significantly above chance level as tested in a one-sample sign test ($N = 68$, $P < 0.05$; Fig. 1d). This demonstrates associative learning between olfactory stimuli and FRU reinforcement. This conclusion is drawn from comparisons of test performance between reciprocal training regimes (AM+/OCT versus AM/OCT+). The overall preference for AM over OCT (Fig. 1b, c) merely leads to an offset of the preference values for the two groups but cannot cause differences in preference values as measured by LI (Fig. 1d). Therefore, LI gives a pure measure of associative learning between olfactory stimuli and FRU reinforcement.

Number of Learning Trials

Three learning trials were sufficient to yield asymptotic levels of learning; specifically, LI differed between the groups trained with either one, two, three, four or eight learning trials (Kruskal–Wallis test: $H_4 = 15.4$, $N = 43$, 72, 57, 44, 68, $P < 0.05$; Fig. 2). LIs were not different from zero when only one or two training trials were given (one-sample sign test: sample sizes as above, NS in both cases; Fig. 2) but learning could be demonstrated for three, four and eight learning trials, as the LIs were significantly different from zero in each case (one-sample sign test: sample sizes as above, $P < 0.05$ in all cases; Fig. 2). Learning after three trials was as good as after eight (Mann–Whitney test: $Z = -0.80$, sample sizes as above, NS; Fig. 2b), but better than after two trials ($Z = -1.90$, sample sizes as above, $P = 0.05$; Fig. 2b). Thus, increasing the number of training trials beyond three did not lead to obvious increments in learning.

Reinforcement Intensity

LI differed between the groups trained with different concentrations of FRU (Kruskal–Wallis test: $H_3 = 15.2$, $N = 69$, 63, 52, 74; $P < 0.05$; Fig. 3). LI was not different from zero when 0.25 or 0.5 mol/litre FRU was used (one-sample sign test: sample sizes as above, NS in both cases; Fig. 3) but learning could be demonstrated for 1 and 2 mol/litre FRU (one-sample sign test: sample sizes as above, $P < 0.05$ in both cases; Fig. 2). Using 1 mol/litre FRU led to learning indistinguishable from using 2 mol/litre (Mann–Whitney test: $Z = -0.75$, sample sizes as above, NS; Fig. 3b), but better than with 0.25 mol/litre ($Z = -2.93$, sample sizes as above, $P < 0.05$; Fig. 3). Thus, increasing reinforcement intensity beyond 1 mol/litre did not lead to substantial increments in learning.

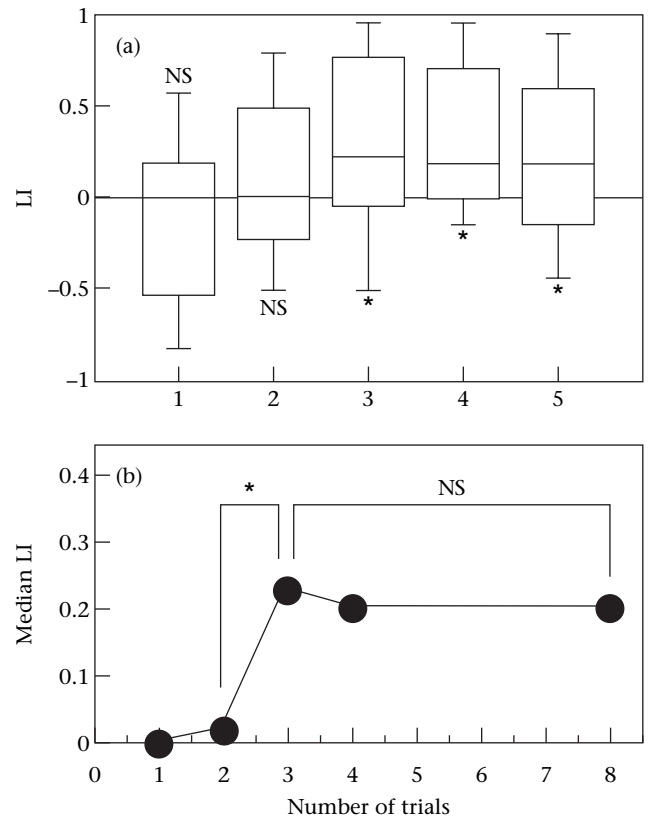


Figure 2. (a) Learning index (LI) with one, two, three, four or eight conditioning trials. (b) Median LIs plotted against the number of trials; note the truncated axis. * $P < 0.05$, one-sample sign tests (a) and Mann–Whitney U tests (b). For an explanation of the box plots, see legend of Fig. 1.

Age of Larvae

LI did not differ between groups aged 4, 5 and 6 days after egg laying ($\bar{X} \pm SD = 84, 108, 132$ h; range cap ± 12 h in each case; Kruskal–Wallis test: $H_2 = 0.9$, $N = 80, 80, 80$, NS; Fig. 4). In each case the LIs were significantly above chance level (one-sample sign test: sample sizes as above, $P < 0.05$ in all cases; Fig. 4). Thus learning ability was not developmentally modulated at 4–6 days after egg laying.

Gender of Larvae

LI did not differ between genders (Mann–Whitney test: $Z = 0.01$, $N_1 = 38$, $N_2 = 39$, NS; Fig. 5). In both cases the LIs were significantly above chance level (one-sample sign test: sample sizes as above, $P < 0.05$ in each case; Fig. 5). Thus learning ability was not modulated by gender.

En Masse Assays

In the en masse assay, the LIs were significantly different from zero (one-sample sign test; $N = 15$, $P < 0.05$; Fig. 6a), showed little scatter, and yielded a median of 0.31.

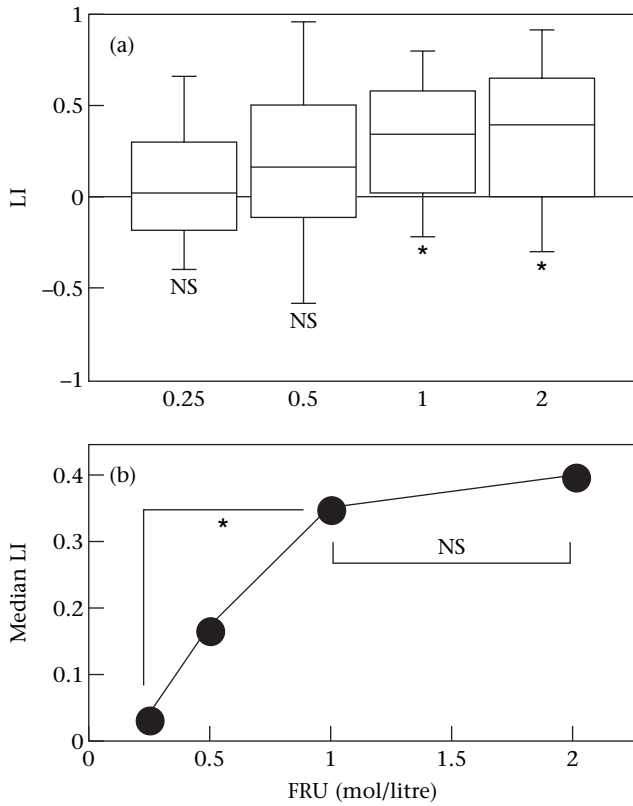


Figure 3. (a) Learning index (LI) with 0.25, 0.5, 1.0, or 2.0 mol/litre of fructose (FRU) for reinforcement. (b) Median LIs plotted against the FRU concentration; note the truncated axis. $*P < 0.05$, one-sample sign tests (a) and Mann-Whitney U tests (b). For an explanation of the box plots, see legend of Fig. 1.

LIs did not differ between genders (Mann-Whitney U test: $U = 111$; $N_1 = N_2 = 15$, NS; Fig. 6b), confirming the above negative results from the individual-animal assay (Fig. 5). For both genders, LIs were above chance level (one-sample sign test: sample sizes as above, $P < 0.05$ in both cases; Fig. 6b).

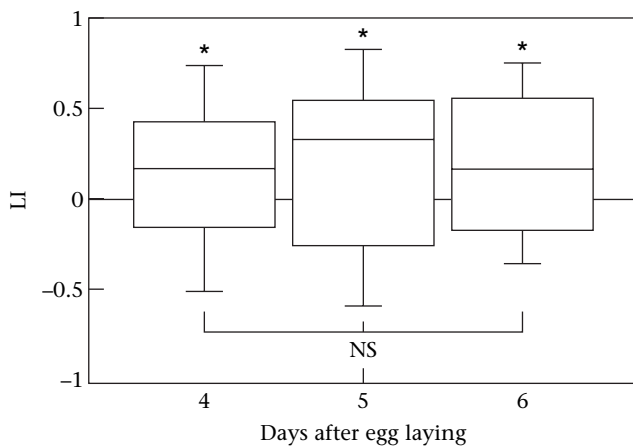


Figure 4. (a) Learning index (LI) for larvae aged either 4, 5, or 6 days after egg laying. $*P < 0.05$ for difference from chance level; one-sample sign tests were used. For a between-group comparison, a Kruskal-Wallis test was used. For an explanation of the box plots, see legend of Fig. 1.

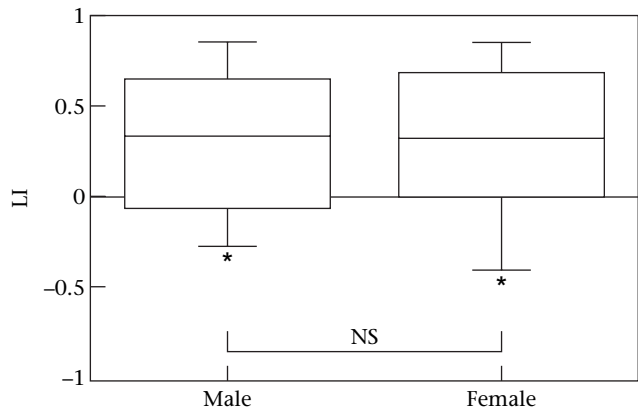


Figure 5. Learning index (LI) for male and female larvae. Larval gender was determined post hoc in the adults. $*P < 0.05$ for difference from chance level. One-sample sign tests were used. To test for between-group differences, a Mann-Whitney U test was used. For an explanation of the box plots, see legend of Fig. 1.

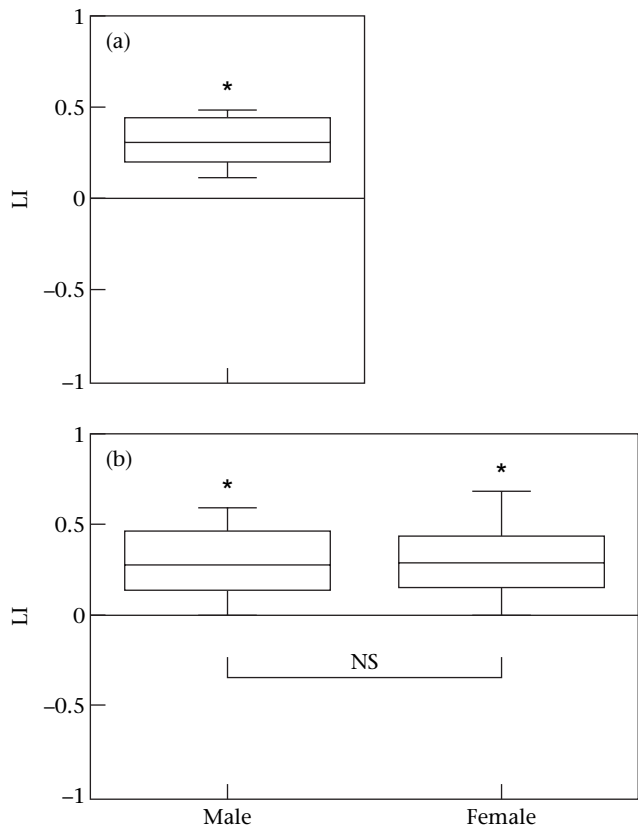


Figure 6. (a) Learning index (LI) from the en masse assay. Preference values were calculated for each set of 30 larvae as the number of larvae on the AM side at 3 min after beginning the test, minus the number on the OCT side, divided by the total number of larvae. Using these preference values, we calculated LIs as explained in the legend of Fig. 1d. (b) LI for male and female larvae. $*P < 0.05$ for difference from chance level. One-sample sign tests were used. For the two-group comparison in (b), a Mann-Whitney U test was used. For an explanation of the box plots, see legend of Fig. 1.

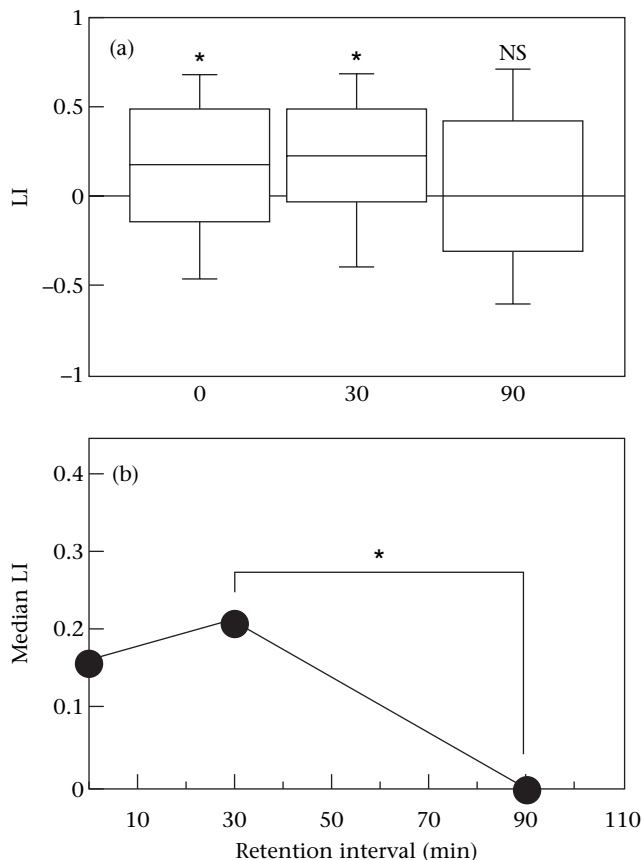


Figure 7. (a) Learning index (LI) with retention intervals of 0, 30, or 90 min. (b) Median LIs plotted against the retention interval; note the truncated axis. * $P < 0.05$, for difference to zero, one-sample sign tests were used. For the between-group comparison in (b), a Mann–Whitney U test was used. For an explanation of the box plots, see legend of Fig. 1.

Memory Stability

Memory retention differed depending on retention interval (Kruskal–Wallis test: $H_2 = 6.07$, $N = 112, 119, 117$; $P < 0.05$; Fig. 7a). For immediate retention and the 30-min retention interval, LIs were significantly above chance level (one-sample sign test: sample sizes as above, $P < 0.05$ in both cases; Fig. 7a). From the retention level at 30 min, memory decayed significantly until 90 min (Mann–Whitney U test: $Z = -2.22$, $N_{30 \text{ min}} = 119$, $N_{90 \text{ min}} = 117$, $P < 0.05$), when scores came down to random levels (one-sample sign test: sample sizes as above, NS; Fig. 7a, b). Thus, memory in our paradigm was fully stable for at least 30 min.

DISCUSSION

Our study is the first detailed parametric analysis of appetitive olfactory learning in individually assayed *Drosophila* larvae. Our main findings are that learning reached an asymptote after three training trials; a concentration of 2 mol/litre of fructose supported the highest learning scores; learning was not modulated by age or gender; en masse assays were feasible and confirmed the lack of

gender differences in learning; and memory was fully stable for at least 30 min.

Versatility of the Assays

Our procedure made it possible to obtain significant learning scores in 250 min of experimentation: about 80 individual animals, 40 for each reciprocal training condition, were needed to calculate 40 LI values, which are sufficient to demonstrate learning. We used batches of eight larvae trained together but tested singly, thus testing eight animals and, on average, yielding four LI values in at most 25 min, including handling. Thus, sufficient data to demonstrate learning can be accumulated after at most 250 min of experimentation (our en masse assay would yield $N = 5$ in the same time, and thus takes about three times as long to yield a sample size of $N = 15$ which should be about sufficient). This makes the current assay versatile for genetic analysis, which typically needs to run multiple genetic controls along with the experimental genotype.

The en masse assay yielded a median LI of 0.37, which is almost twice as large as that in the individual-animal assay (compare Fig. 6a and Figs 1–5). The LIs in our en masse assay are also somewhat higher than the ca. 0.27 found by Dukas (1999). To some extent, the LIs might be higher in the en masse assay than in the individual-animal assay because the score was taken at only one time point, after 3 min, so that in particular an early phase of indecisiveness (see Fig. 1) is not considered. Also, in the en masse assay, no III was used. Thus, a direct, quantitative comparison of the LIs between the en masse assay and the individual-animal assay is of no heuristic value.

From a practical point of view, however, the relatively less variable LIs found in the en masse assay might lend this procedure and mode of data acquisition to experiments comparing different genotypes, as reduced scatter will make it easier to detect differences in LIs between genotypes. The low scatter of LIs in the en masse assay might reflect the fact that each LI already represents a mean of the sampled group of animals. The individual-animal assay, on the other hand, has the advantage that fewer animals are needed, so that behavioural experiments can be more easily combined with physiological approaches (Schmucker et al. 1994; Koh et al. 2000; Fiala et al. 2002; Liu et al. 2003); furthermore, the individual-animal assay needs less experimental time to yield significant learning effects.

Repetitions Needed

We found that three learning trials were enough to yield asymptotic levels of learning in our paradigm (Fig. 2). Honeybees, *Apis mellifera*, also reach asymptotic learning performance after three rewarded trials (Hammer & Menzel 1995), but can learn in a single trial. Nevertheless, the amount of training needed in our paradigm seems modest; this might be related to the evolutionary design of the larva as the feeding stage of the life cycle of the fly; therefore nutritious stimuli might be particularly rewarding to them. This is in line with the observation that

aversive stimuli lack a punishing effect in our paradigms (Gerber et al. 2004; Hendel et al., in press).

Lack of Age and Gender Effect

We found no evidence that larval age modulates learning ability (Fig. 4). In mature adult flies, effects of age on learning are relatively small and specific to the task tested (Le Bourg 2004). However, with better temporal resolution of the age classes or with an extension of the range of age classes maybe into earlier larval stages, it might be possible to uncover an effect of larval age. This is because age classes were defined with a precision of only ± 12 h. If either only the youngest or only the oldest larvae had reduced learning ability, such an effect might have been obscured by the older or younger animals within the same age class. Also, larvae aged < 4 or > 6 days after laying might show a difference in learning; for the latter, it might be interesting to test larvae closer to the larva-to-pupa transition, i.e. wandering-stage larvae, rather than the feeding-stage larvae used here.

We also found no evidence for an effect of gender on learning ability (Figs 5, 6). This is expected because gender does not affect learning ability in adults (e.g. Heisenberg et al. 1985) and because sexual development in the larva has not proceeded beyond the establishment of the gonad imaginal discs.

Memory Span

Appetitive olfactory memory in the larva persisted without decay for at least 30 min after training; after 90 min, memory had fully decayed (Fig. 7). For aversive olfactory memory in the larva, established by pairings of odours with electric shock, Tully et al. (1994) claimed that memory persists even into adulthood. However, the aversive larval learning effect itself (see also Aceves-Piña & Quinn 1979; Heisenberg et al. 1985) in at least two cases could not be replicated (Forbes 1993; F. Python, personal communication).

From a practical standpoint, the ability to detect learning up to 30 min after training makes a number of additional experimental manipulations feasible; these include pharmacological and amnesic treatments and in particular using transgenic expression of a dominant-negative, temperature-sensitive form of the *shibire* gene to turn on and off subsets of neurons in the larval brain (Kitamoto 2001) to study the involvement of these neurons in memory storage and retrieval (reviewed in Heisenberg 2003). Given the technical simplicity of our assay as well as the relatively high speed of data acquisition, such approaches now seem feasible. This hopefully provides the research community with a fruitful experimental system for behavioural neuroscience.

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References

- Aceves-Piña, E. O. & Quinn, W. G. 1979. Learning in normal and mutant *Drosophila* larvae. *Science*, **206**, 93–96.
- Bi, G.-q. & Poo, M.-m. 2001. Synaptic modification by correlated activity: Hebb's postulate revisited. *Annual Reviews of Neuroscience*, **24**, 139–166.
- Dukas, R. 1999. Ecological relevance of associative learning in fruit fly larvae. *Behavioral Ecology and Sociobiology*, **45**, 195–200.
- Fiala, A., Spall, T., Diegelmann, S., Eisermann, B., Sachse, S., Devaud, J.-M., Buchner, E. & Galizia, C. G. 2002. Visualization of olfactory information in projection neurons using genetically expressed cameleon in *Drosophila melanogaster*. *Current Biology*, **12**, 1877–1884.
- Forbes, B. 1993. Larval learning and memory in *Drosophila melanogaster*. Diploma thesis, University of Würzburg.
- Gerber, B., Scherer, S., Neuser, K., Michels, B., Hendel, T., Stocker, R. F. & Heisenberg, M. 2004. Visual learning in individually assayed *Drosophila* larvae. *Journal of Experimental Biology*, **207**, 179–188.
- Hammer, M. & Menzel, R. 1995. Learning and memory in the honeybee. *Journal of Neuroscience*, **15**, 1617–1630.
- Heisenberg, M. 2003. Mushroom body memoir: from maps to models. *Nature Reviews in Neuroscience*, **4**, 266–275.
- Heisenberg, M., Borst, A., Wagner, S. & Byers, D. 1985. *Drosophila* mushroom body mutants are deficient in olfactory learning. *Journal of Neurogenetics*, **2**, 1–30.
- Hendel, T., Michels, B., Neuser, K., Schipanski, A., Kaun, K., Sokolowski, M. B., Marohn, F., Michel, R., Heisenberg, M., & Gerber, B. In press. The carrot, not the stick: appetitive rather than aversive gustatory stimuli support associative olfactory learning in individually assayed *Drosophila* larva. *Journal of comparative physiology (A)*.
- Kitamoto, T. 2001. Conditional modification of behavior in *Drosophila* by targeted expression of a temperature-sensitive *shibire* allele in defined neurons. *Journal of Neurobiology*, **47**, 81–92.
- Koh, Y. H., Gramates, L. S. & Budnik, V. 2000. *Drosophila* larval neuromuscular junction: molecular components underlying synaptic plasticity. *Microscopy Research and Technique*, **49**, 14–25.
- Le Bourg, E. 2004. Effects of aging on learned suppression of photopositive tendencies in *Drosophila melanogaster*. *Neurobiology of Aging*, **25**, 1241–1252.
- Liu, L., Yermolaieva, O., Johnson, W. A., Abboud, F. M. & Welsh, M. J. 2003. Identification and function of thermosensory neurons in *Drosophila* larvae. *Nature Neuroscience*, **6**, 267–273.
- Martin, S. J., Grimwood, P. D. & Morris, R. G. 2000. Synaptic plasticity and memory: an evaluation of the hypothesis. *Annual Reviews of Neuroscience*, **23**, 649–711.
- Phelps, C. B. & Brand, A. H. 1998. Ectopic gene expression in *Drosophila* using GAL4 system. *Methods: A Companion to Methods in Enzymology*, **14**, 367–379.

- Python, F. & Stocker, R. F.** 2002. Adult-like complexity of the larval antennal lobe of *D. melanogaster* despite markedly low numbers of odorant receptor neurons. *Journal of Comparative Neurology*, **445**, 374–387.
- Rescorla, R. A.** 1988. Behavioral studies of pavlovian conditioning. *Annual Reviews of Neuroscience*, **11**, 329–352.
- Scherer, S., Stocker, R. F. & Gerber, B.** 2003. Olfactory learning in individually assayed *Drosophila* larvae. *Learning and Memory*, **10**, 217–225.
- Schmucker, D., Su, A. L., Beerman, B., Jackle, H. & Jay, D. G.** 1994. Chromophore-assisted laser inactivation of patched protein switches cell fate in the larval visual system of *Drosophila*. *Proceedings of the National Academy of Sciences, U.S.A.*, **91**, 2666–2668.
- Sokolowski, M. B.** 2001. *Drosophila*: genetics meets behavior. *Nature Reviews in Genetics*, **2**, 879–890.
- Stocker, R. F.** 2001. *Drosophila* as a focus in olfactory research: mapping of olfactory sensilla by fine structure, odor specificity, odorant receptor expression, and central connectivity. *Microscopy Research and Technique*, **55**, 284–296.
- Tully, T., Cambiazo, V. & Kruse, L.** 1994. Memory through metamorphosis in normal and mutant *Drosophila*. *Journal of Neuroscience*, **14**, 68–74.