The Journal of Experimental Biology 207, 179-188
Published by The Company of Biologists 2004
doi:10.1242/jeb.00718

Visual learning in individually assayed *Drosophila* larvae

B. Gerber[1,2,*], S. Scherer[2], K. Neuser[1], B. Michels[1], T. Hendel[1], R. F. Stocker[2] and M. Heisenberg[1]

[1]*University of Würzburg, Department of Genetics and Neurobiology, Biocentre Am Hubland, D 970 74 Würzburg, Germany* and [2]*University of Fribourg, Department of Biology and Program in Neuroscience, Ch. du Musée 10, CH 1700 Fribourg, Switzerland*

*Author for correspondence (e-mail: bertram.gerber@biozentrum.uni-wuerzburg.de)

Accepted 23 September 2003

Summary

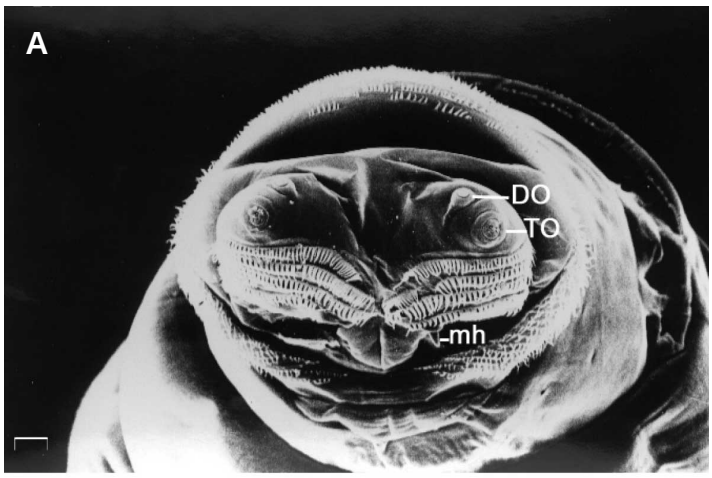
An understanding of associative learning is facilitated if it can be analyzed in a simple animal like the fruit fly *Drosophila*. Here, we introduce the first visual associative learning paradigm for larval *Drosophila*; this is remarkable as larvae have an order of magnitude fewer neurons than adult flies. Larvae were subjected to either of two reciprocal training regimes: Light+/Dark– or Light–/Dark+. Subsequently, all larvae were individually tested for their preference between Light *versus* Dark. The difference between training regimes was therefore exclusively which visual situation was associated with which reinforcer; differences observed during the test thus reflected exclusively associative learning. For positive reinforcement (+) we used fructose (FRU), and for negative reinforcement (–) either quinine or sodium chloride (QUI, NaCl). Under these conditions, associative learning could be reproducibly observed in both wild-type strains tested. We then compared the effectiveness of training using differential conditioning, with both positive and negative reinforcement, to that using only positive or only negative reinforcement. We found that FRU only, but neither QUI nor NaCl, was in itself effective as a reinforcer. This is the first demonstration of appetitive learning in larval *Drosophila*. It is now possible to investigate the behavioral and neuronal organization of appetitive visual learning in this simple and genetically easy-to-manipulate experimental system.

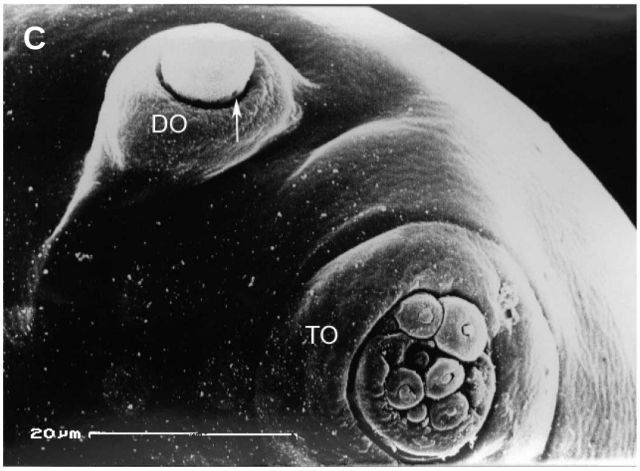
Key words: *Drosophila*, larva, vision, learning, taste.

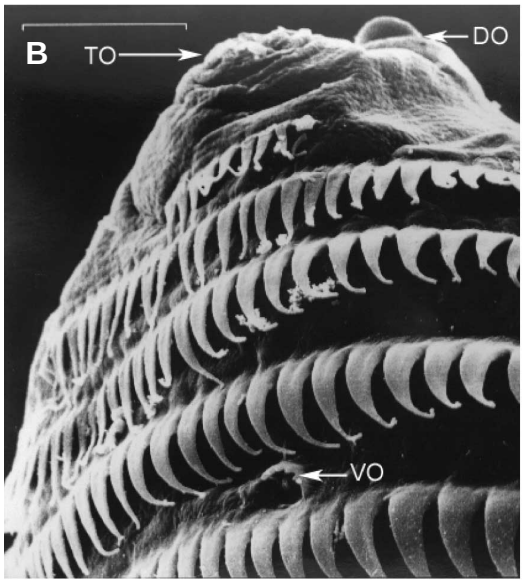
Introduction

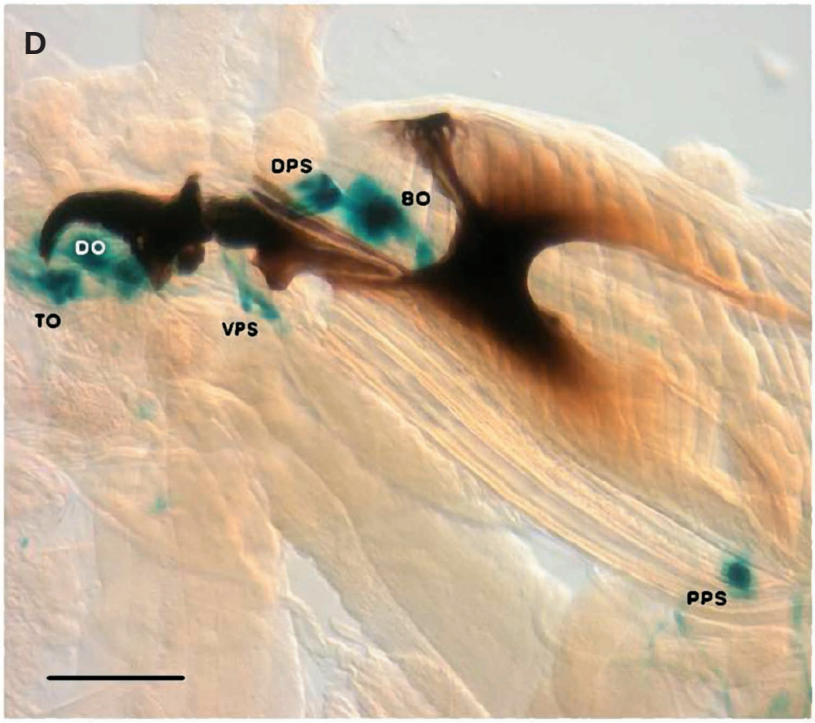
Associative learning enables animals to prepare for important events. An understanding of such associative learning is facilitated if it can be studied in a simple system and at many levels of analysis. The fruit fly *Drosophila melanogaster* is a model system that can meet these demands. Its brain contains 3–6 orders of magnitude fewer neurons compared to mammals. It also offers many possibilities for relating behavioral analysis to genetics, molecular biology and electrophysiology (Sokolowski, 2001). Importantly, recent advances in manipulating the *Drosophila* brain using transgenic techniques (Phelps and Brand, 1998; Kitamoto, 2001) were combined with behavioral analysis to make this system suitable for an integrative approach to associative function (Heisenberg, 2003; Waddell and Quinn, 2001; Zars, 2000). Furthermore, flies and mammals share many homologous genes (Rubin et al., 2000), suggesting that molecular mechanisms underlying behavioral plasticity might be shared. Thus, the study of *Drosophila* may also provide educated guesses for research on mammals.

Recently, a number of studies have focused on larval *Drosophila*, probably because they have ten times fewer neurons than adults (e.g. Busto et al., 1999; Hassan et al., 2000; Heimbeck et al., 1999; Liu et al., 2003a,b; Python and Stocker, 2002a,b; Scott et al., 2001). For example, comparing larva to adult, the number of receptor neurons within each hemisphere is 12 *versus* 6000 for vision, 21 *versus* 1300 for olfaction and 80 *versus* 650 for taste (Stocker, 1994, 2001) (for an overview, see Fig. 1A–E). Relatively little, however, is known about associative learning in *Drosophila* larvae. This is unfortunate, as our knowledge concerning the physiological mechanisms of synaptic plasticity, which are commonly thought to underlie behavioral plasticity, largely derives from experiments in the larva (Koh et al., 2000). Previous learning experiments on larval olfactory learning were performed using *en masse* assays (Aceves-Piña and Quinn, 1979; Heisenberg et al., 1985; Tully et al., 1994; Dukas, 1998), which preclude a combined behavior and physiology approach, because too many animals are needed to yield learning effects. In this study, therefore, we used individually assayed larvae and established a visual learning paradigm. In contrast to Aceves-Piña and Quinn (1979), Heisenberg et al. (1985) and Tully et al. (1994), we used gustatory stimuli rather than electric shock as reinforcement, largely because the reproducibility of electric shock learning is compromised (Forbes, 1993; F. Python, personal communication); also, gustatory reinforcement, rather than electric shock, seems biologically relevant for larval *Drosophila*.


A
DO
TO
mh


C
DO
TO
20 µm


B
TO
DO
VO


D
DPS
BO
DO
TO
VPS
PPS

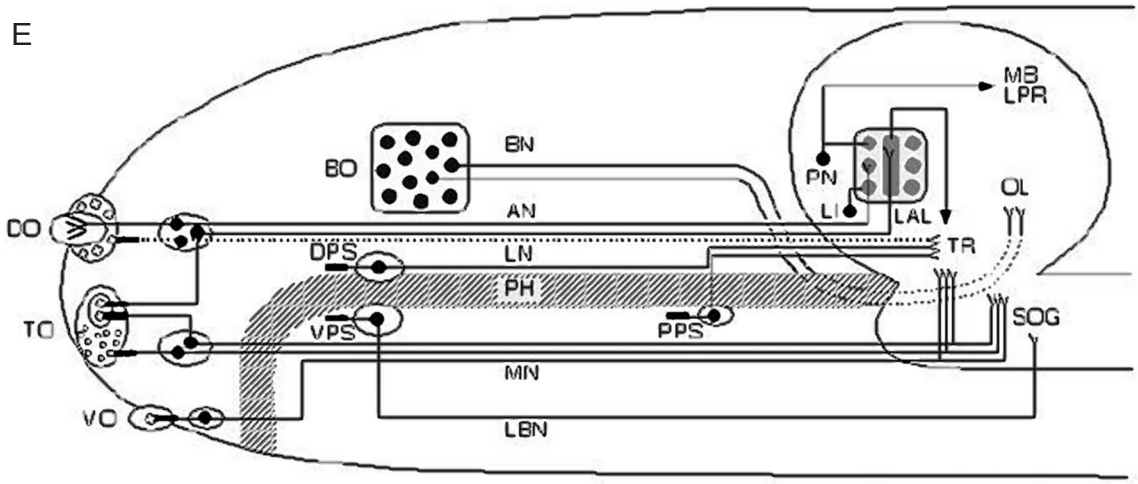

E
MB
LPR
BO
BN
PN
OL
AN
LAL
DO
DPS
LN
TR
PH
TO
VPS
PPS
SOG
MN
VO
LBN

Fig. 1. (A–C) SEM images of the external chemosensory organs of the larval head. (A) Frontal overview; (B) enlarged view of the ventral organ (VO) and (C) dorsal (DO) and terminal (TO) organs. VO and TO probably have gustatory functions, whereas the DO serves both gustatory and olfactory functions. MH, mouth hook. The arrow in C points to one of the pores of the dome (DM). Scale bars, 20 μm. (D) Expression of the lacZ reporter in the Gal4 driver line MJ94, showing the position of the larval eye – the Bolwig's organ (BO) – in front of the cephalopharyngeal skeleton (dark brown). Further reporter expression also visualizes the positions of DO, TO and the dorsal, ventral and posterior pharyngeal gustatory sensilla (DPS, VPS, PPS). Scale bar, 100 μm. (E) Wiring diagram showing the central projections of the head sensory organs to the optic lobe (OL) tritocerebrum (TR), suboesophageal ganglion (SOG) and antennal lobe (AL) (modified from Python and Stocker, 2002a). AN, BN, LN, MN and LBN: antennal, Bolwig, labral, maxillary and labial nerves, respectively. LI, PN, local interneurons of the AL, projection neurons; LPR, lateral protocerebrum; MB, mushroom bodies; PH, pharynx.

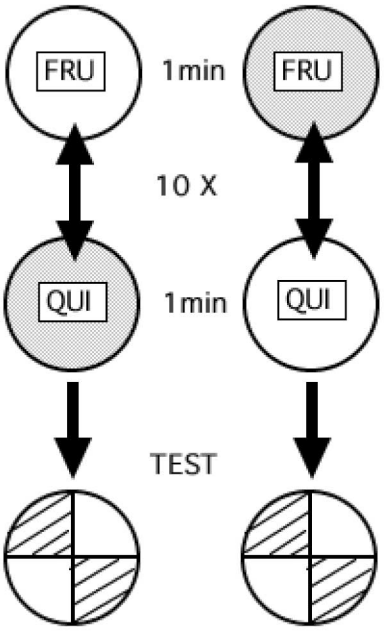


Fig. 2. Sketch of the experimental protocol for the learning experiments; for details see text. Please note that within each treatment condition, e.g. Light+/Dark–, half of the animals were trained with Light+ as the first trial and half of the animals with Dark– as the first trial.

Using visual stimuli and gustatory reinforcement, we can demonstrate for the first time visual associative learning in *Drosophila* larvae; furthermore, this study is the first to demonstrate appetitive larval learning. The current paradigm, together with its concurrently developed olfactory companion study (Scherer et al., 2003), thus opens up the possibility of comparing the organization of visual and olfactory memories in a simple and genetically easy-to-manipulate nervous system.

Materials and methods

Principle of training

In all learning experiments, animals underwent one of two reciprocal training regimes (for a sketch, see Fig. 2). They either received positive reinforcement (fructose) in light and negative reinforcement (either quinine or sodium chloride) in darkness (Light+/Dark–); or they were trained reciprocally (Light–/Dark+). Subsequently, in the absence of any reinforcer, animals were individually tested for their choice between Light and Dark. During this test, systematic differences between individuals subjected to the reciprocal training regimes were exclusively due to associative learning. This conclusion is compelling as individuals from both training regimes had identical exposure to both light and dark and to the reinforcers; what differed was exclusively the contingency between visual condition and reinforcer.

Larvae

We used Canton-S wild-type strains from two stock collections, either Fribourg (CS-F) (Experiments 1, 3), or Würzburg (CS-W) (Experiments 2, 4, 5). All flies were kept in the Würzburg facility in mass culture maintained at 24°C, 60–70% relative humidity and subjected to a 14 h:10 h light:dark cycle. Daily, adult flies were transferred from their current into a fresh food vial where they were allowed to lay eggs for 24 h. At 115 h after commencement of the egg-laying period, experiments were begun; experimental larvae were therefore aged 91–115 h, in some cases even 122 h after egg lay (AEL). In a companion study (B. Gerber, S. Scherer, M. Kretz, R. F. Stocker, and M. Heisenberg, manuscript in preparation), we found no effect of age (i.e. 67–91 h, 91–115 h or 115–139 h AEL) on the larval photoresponse (but see Sawin-McCormack et al., 1995).

Our procedure of larval staging is admittedly coarse; still, exact staging does not seem to lead to altered or to less variable learning scores, at least in the olfactory version of our paradigm (T. Hendel, unpublished data).

On experimental days, a spoonful of food substrate containing larvae was taken and transferred to a small glass vial. From there, individual animals were removed using a paintbrush, briefly washed in tapwater, and immediately placed into the experimental arena. Thus, in contrast to the procedures used for larval harvest in mass assays, animals were taken exclusively from the food, not from the wall, in order to reduce the likelihood of harvesting wandering stage larvae.

Experimental conditions

Test plates

Agarose (1%; electrophoresis grade, Roth, Karlsruhe, Germany) was boiled in a microwave oven and allowed to cool down for 30 min, with constant gentle stirring. Petri dishes (9 cm inner diameter; Sarstedt, Nümbrecht, Germany) were then filled with a thin layer of agarose. The agarose was allowed to solidify for 20 min under a protective mesh. Then, lids were put on the plates to avoid drying out and plates were

stored at room temperature for use as test plates until the following day.

Training plates

As a potentially negative gustatory reinforcer we used quinine hemisulfate (QUI; purity 92%; Sigma, Steinheih, Germany) or sodium chloride (NaCl; purity 99%; Roth, Karlsruhe, Germany) and as a potentially positive reinforcer, fructose (FRU; purity 99%; Sigma). These reinforcers were added to the agarose 10 min after boiling to reach final concentrations of 0.2% QUI, 4 mol l^{-1} NaCl and 1 mol l^{-1} FRU. Petri dishes with 5 cm inner diameter were used to prepare these training plates.

The experimental room was dark except for the experimental light sources; room temperature ranged from 21–25°C. We used cold-light sources with a homogeneous emission spectrum but no UV or IR emission (Intralux 6000 in combination with the '5″ backlight' light table; VOLPI, Schlieren, Switzerland). The Petri dishes were placed in Perspex trays such that the bottom of the dish was elevated 5 mm above the surface of the light table. To shield parts of the Petri dish from light, we inserted black cardboard glued to a transparent foil between the light source and the tray. The cardboard was 3 mm above the light source and 2 mm below the Petri dish. Between the light source and the cardboard, a 1 mm thick aluminum shield was inserted to prevent heating of the Petri dishes and of the cardboard covers. Thus, the 'layers' of the setup were: light table, air, aluminum shield, tranparent foil with/without cardboard, air, Petri dish.

For training, the light table was divided into an illuminated and a dark half, so that the entire training plates could be placed onto either the illuminated or the dark part of the light table. To generate a choice situation during test, we used an assay with two quadrants illuminated and two quadrants dark ('X-plate').

Training and test in Experiments 1, 2

Animals underwent either Light+/Dark– or Light–/Dark+ training. Each training trial lasted 1 min. For one half of the animals we started with Dark, for the other half with Light; also, we started with the positive reinforcer for half of the animals, and with the negative reinforcer for the other half. This procedure, together with the reciprocal design of the experimental regimes, precludes non-associative contributions to test performance.

Three larvae were transferred to the center of a training plate using a paintbrush; the training plate contained one of the reinforcers and was exposed to one of the visual conditions (e.g. Light+). Then, the lid was closed and the larvae were allowed to freely move for 1 min. The larvae were then immediately transferred to a second assay plate containing the other reinforcer and exposed to the alternative visual condition (Dark–). This cycle was repeated ten times. Fresh plates were used for each trial.

After training, each larva was individually tested for its light preference in the X-plate assay (see below for details); this was done on a separate, fresh test plate, which did not contain any reinforcer. Thus, animals were trained in small groups of three, but tested as individuals.

Animals from both training regimes were trained alternately. On half the days, we started with animals from the one, and on the other half of the days with animals from the other training regime.

To avoid bias, the identity of the reinforcer was coded before experiments, so that the experimenter was 'blind' with respect to the identity of the reinforcers; these identities were decoded only after the experiment.

Modifications for 'absolute' conditioning in Experiment 5

In Experiment 5, three of the five pairs of reciprocal groups were trained in an 'absolute conditioning' procedure. That is, in these groups only one reinforcer was used during training. In an attempt to speed up data acquisition, all animals in Experiment 5 were trained as groups of eight animals instead of three, and tested individually in a down-sized version of the X-plate using Petri dishes with 5 cm inner diameter. Furthermore, we used 2 mol l^{-1} instead of 1 mol l^{-1} FRU. All other details were as specified above.

Behavioral measures and data analysis

For the test, each larva was individually placed in the middle of a test plate, the lid was closed and the larva could then freely move between illuminated and dark quadrants. The position of the larva, as defined by the position of its mouth hooks, was scored every 10 s for 5 min as being in Light or Dark.

The test performance is presented in three steps, described below and illustrated in Figs 3A–C and 4A–C.

(A) For a time-resolved description of the animals' performance, we present the percentage of larvae in Dark for each time point as:

$$\%\ \text{in Dark} = (\text{animals in Dark}/\text{total animals}) \times 100\ .$$

Thus, a value of 100% indicates that all larvae were recorded in a Dark quadrant, 0% indicates all were in a Light quadrant and 50% indicates equal distribution.

(B) We calculated a preference value for each individual as:

$$\text{Dark PREF} = (\text{counts in Dark} - \text{counts in Light}) / \text{total counts}\ .$$

Thus, positive values indicate a dark preference of a given individual and negative values a light preference. In this calculation, temporal resolution is lost. The PREF values of the animals from a given training regime are then represented by box plots. To statistically test for associative learning, we compared these dark preference values between training regimes; as individuals from both training regimes were trained and tested alternately, we could pair them and used the Wilcoxon signed rank test for paired samples; all conclusions remain unaltered if Mann–Whitney *U*-tests or either paired or unpaired *t*-tests are used. As argued below, the comparison of reciprocally trained animals and hence the conclusion regarding associative learning is unaffected by baseline preferences for Dark or Light.

(C) To quantify learning performance and to compare

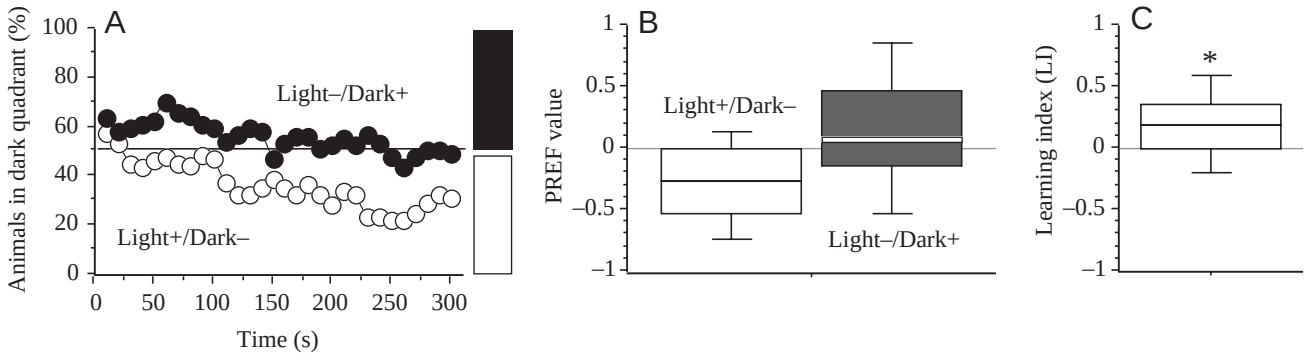


Fig. 3. Experiment 1, showing the test performance of individually assayed wild-type CS-F larvae, which had received either of two reciprocal training regimes: Light–/Dark+ (filled symbols) or Light+/Dark– (open symbols). (A) Percentage of animals located in a covered ('dark') quadrant of an X-plate photoresponse assay. Animals were observed every 10 s for 5 min. The 50% line indicates random distribution. (B) For each animal, a PREF value was calculated. Positive values indicate dark preference and negative values, light preference. The box plot represents the median as the middle line and 10 and 90 and 25 and 75% quantiles as whiskers and box boundaries, respectively. (C) A learning index (LI) was calculated for each pair of animals which underwent either Light–/Dark+ or Light+/Dark– training by subtracting the animals PREF values and dividing the result by two. Positive LIs indicate associative learning. The box plot represents the median as the middle line and 10 and 90, and 25 and 75% quantiles as whiskers and box boundaries, respectively. *P<0.0001 (significantly above chance level). Sample sizes are N=75, 75 for filled and open symbols, respectively; N_{LI}=75.

learning performance between experimental conditions, we calculated a learning index (LI) for the paired individuals as:

$$LI = (PREF^{Light–/Dark+} – PREF^{Light+/Dark–}) / 2 .$$

Thus, positive values indicate associative learning, in that FRU acts as positive and/or QUI or NaCl as negative reinforcer. For a statistical comparison of LIs against zero, we used the one-sample sign test; for multiple and two-group comparisons of LIs we used Kruskal–Wallis and *U*-tests, respectively. If an animal had to be discarded for technical reasons, no LI could be calculated; thus, the sample size for the LI (N_{LI}) may be reduced.

If a larva left the agarose and climbed onto the lid of the Petri dish before the end of the 5 min observation period, data collection for that animal was stopped at that time point.

Tests for sensitization in Experiment 3

To test whether gustatory stimuli like FRU, QUI or NaCl can non-associatively modulate the light response, we tested the light response in the presence of these stimuli; procedures and data analysis were as detailed above for the test, except that (i) no training was given, (ii) in different sets of individuals, the test plates were made from PURE agarose or in addition contained gustatory stimuli. The first part of Experiment 3 (Fig. 5A,B) was designed to match the gustatory stimuli used in Experiment 1, so we used 1 mol l^{-1} FRU and 0.2% QUI and performed the experiment with CS-F. In addition, we used 1 mol l^{-1} sucrose (SUC; purity 99%; Roth) and 2 mol l^{-1} NaCl (purity 99%; Roth). In the second part of Experiment 3 (Fig. 5C), we wanted to match the conditions for Experiment 4, and therefore used 2 mol l^{-1} FRU and 4 mol l^{-1} NaCl as well as CS-W in the down-sized X-plate assay.

Scanning electron microscopy and histology

For scanning electron microscopy (SEM), larvae were rinsed five times in water, cooled to immobility, and the last segment cut off. Then, larvae were fixed overnight in 6,25% glutaraldehyde with 0.1 mol l^{-1} Sörensen phosphate buffer (pH 7.4). Fixed specimens were washed five times in buffer for 5 min each and dehydrated through a graded series of acetone. After critical-point drying in CO_2 (BALTEC CPD 030; Schalkshühle, Germany), larvae were mounted on a table and sputtered with Pt/Pd (BALZERS UNION sputter; Schalkshühle, Germany). Specimens were viewed using a scanning electron microscope (Zeiss DSM 962, Oberkochen, Germany).

To visualise larval neuroanatomy, the GAL4 driver line MJ94 (Joiner and Griffith, 1999) was crossed with UAS-lacZ (Brand and Perrimon, 1993). F_1 third instar larvae were dissected in Millonig's buffer, fixed in 1% glutaraldehyde (in Millonig's), washed and stained for β-galactosidase activity with 5–10 mg X-Gal ml^{-1} dimethyl sulphoxide (DMSO).

Results

Experiment 1

Drosophila larvae were trained in a visual learning experiment (for a sketch, see Fig. 2) and then tested for their visual preferences. As shown in Fig. 3, animals that had received Light–/Dark+ training show a higher dark preference than animals which had received Light+/Dark– training (Fig. 3B; P<0.0001, Z=–4.6). This difference can be quantified by a median learning index (LI) of 0.20, which is significantly above chance level (Fig. 3C; P<0.0001). These results must lead to the conclusion that individually assayed *Drosophila* larvae show associative learning between visual stimuli and gustatory reinforcement. This is because the conclusion is drawn from comparisons between reciprocal training regimes (Light+/Dark– *versus* Light–/Dark+). The relationship between

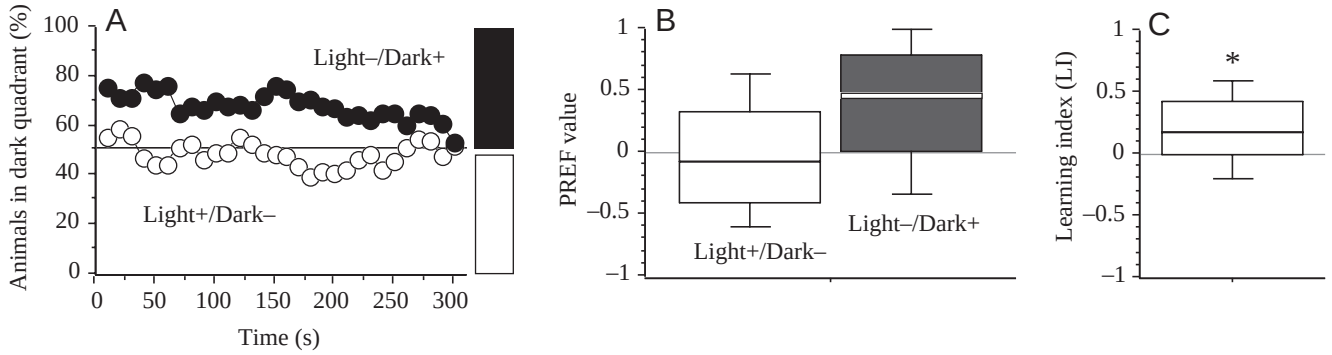


Fig. 4. Experiment 2, which is a repetition of Experiment 1, but uses the CS-W wild-type strain. All other details as in Fig. 1. Sample sizes are N=103, 110 for filled and open symbols, respectively; N_{LI}=102.

visual stimuli and reinforcement is the only difference between these training regimes, so only associative learning can account for the differences seen during the test. Further, this conclusion is unaffected by any preference for Dark or Light; such preferences merely lead to an offset of preference values for both groups but cannot cause differences in test performance between groups as measured by the LI values. Therefore, the conclusion that larval *Drosophila* form associations between visual stimuli and gustatory reinforcement is compelling.

Experiment 2

Visual learning also occurs in another wild-type strain, as shown in Fig. 4 for CS-W. After Light–/Dark+ training, animals are more often observed in a dark quadrant (Fig. 4A) and show a higher dark preference than animals that had received Light+/Dark– training (Fig. 4B; P<0.0001, Z=–5.7); furthermore, the median LI of 0.18 is significantly above chance level (Fig. 4C; P<0.0001).

Interestingly, although animals from CS-F and CS-W strains differ in the time course of performance and in overall dark preference (Figs 3A, 4A), the associative learning effect as measured by the LI is quite similar (Figs 3C, 4C; U=3738; P=0.79).

Experiment 3

Next, we report a sensitization experiment. This is interesting because the above learning experiments were designed to provide a pure measure of associative learning; i.e. any contribution of non-associative learning to the LI (e.g. sensitization) is precluded. The fact that sensitization cannot contribute to the LI does not, however, mean that sensitization cannot occur. For example, FRU might increase overall dark preference (‘stay in this substrate’), whereas NaCl or QUI might have the opposite effect. We specifically asked whether gustatory stimuli might have non-associative effects on the visual response. This is not the case, as the photoresponse is statistically indistinguishable on PURE agarose and in the presence of 1 mol l^{-1} FRU, 1 mol l^{-1} SUC, 2 mol l^{-1} NaCl, or 0.2% QUI (Fig. 5B; P=0.40, H=4.04, d.f.=4). Thus, in CS-F the same gustatory stimuli that can support associative visual learning (Experiment 1; 1 mol l^{-1} FRU and/or 0.2% QUI) do not modulate the photoresponse in a non-associative way.

Experiment 4

We repeated this sensitization experiment under conditions that match the following learning experiment, which used CS-W doubled concentrations of FRU and NaCl (Experiment 5, Fig. 6) and a down-sized version of the X-plate assay. We compared the photoresponse on PURE agarose with the photoresponse on 2 mol l^{-1} FRU and 4 mol l^{-1} NaCl and found no statistically reliable differences (Fig. 5C; P=0.70, H=0.68, d.f.=2). Thus, even 2 mol l^{-1} FRU and 4 mol l^{-1} NaCl do not modulate the photoresponse in a non-associative way. This underlines the purely associative interpretation of the LIs.

Experiment 5

As next step, the reinforcement effectiveness of FRU, QUI, and NaCl was investigated. As shown before, the combination of FRU and QUI could be used effectively for reinforcement (Experiments 1, 2). This leaves open the question of whether FRU or QUI alone would be sufficient to support learning. Thus, using CS-W, we found that the combination FRU/QUI and FRU alone could both effectively support associative learning (Fig. 6; P<0.0001 and P<0.005, respectively); QUI alone, however, did not (Fig. 6; P=0.10). Interestingly, the LI values for the combination FRU/QUI and for FRU alone were statistically indistinguishable (Fig. 6; P=0.42, U=6837.5). Thus, FRU but not QUI is an effective reinforcer for visual associative learning in the *Drosophila* larva.

The same results emerged for 4 mol l^{-1} NaCl. That is, the combination FRU/NaCl effectively supported associative learning (Fig. 6; P<0.05) whereas NaCl alone did not (Fig. 6; P=0.49). Also, the LI values for the combination FRU/NaCl and for FRU alone were indistinguishable (Fig. 6; P=0.63, U=6578.0). These results confirm that FRU is an effective reinforcer for visual associative learning in the *Drosophila* larva and suggest that NaCl is not, and are backed up by a statistically significant overall difference between the five groups (Fig. 6; P<0.05, H=18.75, d.f.=4).

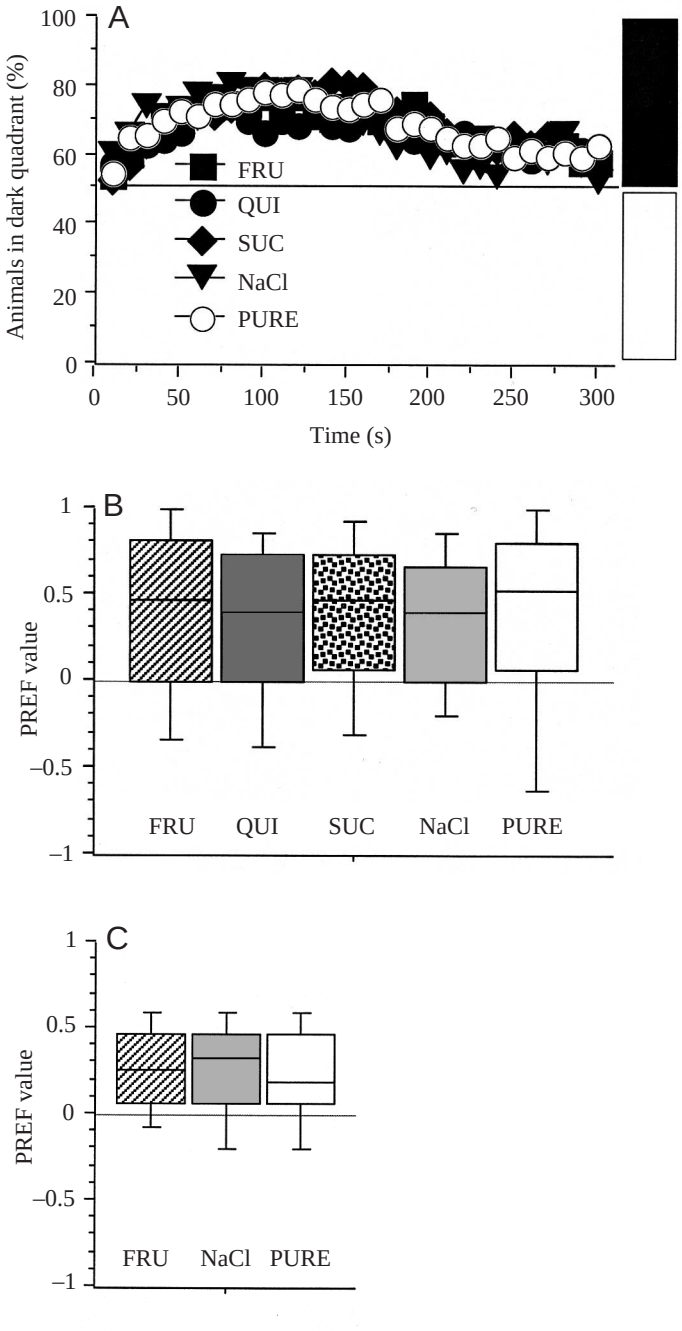


Fig. 5. Experiments 3 and 4, showing the photoresponse of individually assayed larvae, tested on either PURE agarose or on agarose plates containing gustatory stimuli. (A) Percentage of CS-F larvae located in a covered ('dark') quadrant in Experiment 3. Animals were observed every 10 s for 5 min in an X-plate photoresponse assay. The 50% line indicates random distribution. Animals were tested on either fructose (FRU; 1 mol l^{-1}), quinine hemisulfate (QUI; 0.2%), sucrose (SUC; 1 mol l^{-1}), or NaCl (2 mol l^{-1}). (B) For each animal, a PREF value was calculated; positive values indicate dark preference and negative values, light preference. The box plot represents the median as the middle line and 10 and 90, and 25 and 75% quantiles as whiskers and box boundaries, respectively. No significant difference between the PREF values was found. Sample sizes were *N*=150, 150, 142, 146, and 144 for FRU, QUI, SUC, NaCl and PURE, respectively. (C) Experiment 4 is a repetition of Experiment 3, but using animals from the CS-W wild-type strain and employing the down-sized X-plate assay. PREF values were tested on either PURE agarose or on agarose plates, containing FRU (2 mol l^{-1}) or NaCl (4 mol l^{-1}). Sample sizes from left to right, *N*=88, 88, 72.

Discussion

Utility of an individual assay

Using individually assayed larvae, we provide the first evidence for visual learning in *Drosophila* larvae to date. This had been thought impossible to do because inter-individual variability was presumed to be too high (Dukas, 1998).

The current paradigm requires relatively few (20 trials) and short (20 min) training with few (approx. 70) animals, and is similar to a concurrently developed olfactory learning paradigm (Scherer et al., 2003). It requires, however, about an order of magnitude fewer animals than needed for adult olfactory learning using *en masse* assays. The short training time and the need for only a few animals might make this system suitable for electrophysiological approaches (Koh et al., 2000), *in vivo* imaging (Fiala et al., 2002; Liu et al., 2003b), and approaches combined with the MARCM technique (Lee and Luo, 2001), or laser ablation (Schmucker et al., 1994). The cellular simplicity of the larval nervous system will hopefully facilitate these kinds of analyses.

Learning is purely associative

The associative nature of the learning process was ensured by a traditional reciprocal training design. That is, individuals from the two reciprocal training regimes (Light–/Dark+ and Light+/Dark–) have identical experiences with visual stimuli and reinforcer; what is different is exclusively the contingency between them. As we have shown that test performance depends on this contingency (Figs 3, 4, 6), the conclusion regarding associative learning is compelling.

The maximal median LI found in our study is 0.2; this is somewhat less but in the same range as found in three other assays: (i) the concurrently developed olfactory version of this paradigm (Scherer et al., 2003), (ii) larval electric shock olfactory learning with *en masse* assays (Heisenberg et al., 1985; Tully et al., 1994; see, however, Forbes, 1993) and (iii) appetitive olfactory learning in adults using sucrose as reinforcer (Borst, 1983; Heisenberg et al., 1985; Tempel et al., 1983; Schwaerzel et al., in press). Thus, it seems that LI values of about 0.2 are what one can expect for associative learning in larvae and for appetitive learning in adults.

No evidence for a non-associative modulation of the photoresponse

As argued above, the experimental design precludes any non-associative contribution to the LI values. Nevertheless, we tackled the question of whether gustatory stimuli may non-associatively modulate the photoresponse (i.e. the PREF values). For example, FRU might appetitively sensitize larvae and lead to an increased dark preference ('this tastes good –

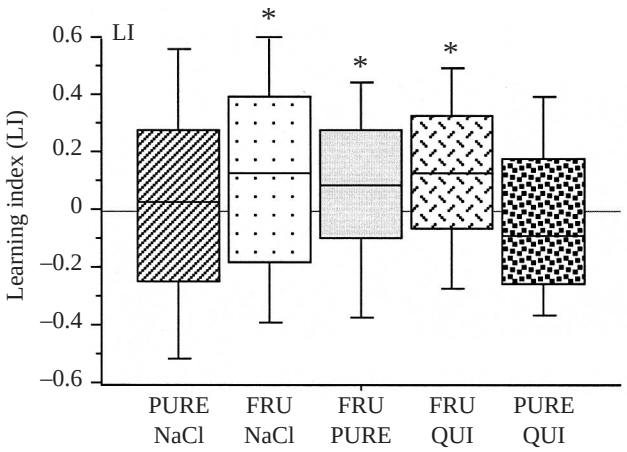


Fig. 6. Experiment 5, showing the learning performance as measured by the LI values of individually assayed CS-W larvae from five learning experiments. Experiments use either NaCl only (PURE/NaCl), a combination of FRU and NaCl (FRU/NaCl), FRU only (FRU/PURE), a combination of FRU and QUI (FRU/QUI), or QUI only (PURE/QUI) as reinforcers. Positive LI values indicate associative learning. The box plot represents the median as the middle line and 10 and 90, and 25 and 75% quantiles as whiskers and box boundaries, respectively. *P<0.05. The test was performed in a down-sized X-plate assay. Please note the truncated axis. Sample sizes are from left to right N_{LI}=108, 123, 111, 131, 117.

go into this substrate'), whereas QUI or NaCl might decrease dark preference ('this tastes horrible – get out of this substrate'). In two series of experiments, we did not find evidence for any such non-associative effect (Fig. 5). This conclusion is in line with the result of Scherer (2002), who found that prior exposure to aqueous solutions of FRU or NaCl does not modulate the photoresponse; it is further consistent with the finding that the olfactory response is also not modulated by the presence of FRU, QUI, or NaCl (Hendel, 2003).

The carrot, not the stick?

The literature on *Drosophila* learning, including larval learning, is largely concerned with aversive reinforcers of almost life-threatening intensity, heat and electric shock being used most frequently (Heisenberg, 2003; Waddel and Quinn, 2001; Zars, 2000). The implicit rationale seems to be that *Drosophila* are stupid and therefore one has to get tough on them.

To our surprise, we demonstrate here that only FRU, not QUI and not NaCl, is a potent reinforcer. This matches recent results from olfactory learning that also indicate that FRU, but not QUI and not NaCl, is an effective reinforcer (Hendel, 2003); also, in adult flies Le Bourg and Buecher (2002) observed QUI to be ineffective as a reinforcer in visual learning. Thus, although QUI and NaCl can well be perceived by the larvae (Heimbeck et al., 1999; Hendel, 2003), appetitive, rather than aversive, gustatory reinforcement seems to be effective. Given the biology of larval *Drosophila* as feeding stages, appetitive reinforcement with FRU seems to meet the larva's biological obsessions; in this respect, *Drosophila* larvae might be regarded as similar to the honeybee forager with its proverbial desire for nectar. Thus, appetitive gustatory reinforcers seem biologically plausible and, in this sense, gentle.

Beyond this ultimate argument, possible proximate reasons for the negative results concerning aversive gustatory stimuli may be manyfold. For example, it might be that pharyngeal, rather than external, gustatory sensilla drive the modulatory, internal reinforcement pathway (see Fig. 1 for an overview). Suppose larvae swallow crumbs of FRU-containing agarose, but only to a lesser extent, QUI or NaCl-containing agarose; FRU rather than QUI and NaCl could thus drive pharyngeal gustatory sensilla and hence an internal reinforcement signal. Maybe because of this compromised access, QUI and NaCl are not effective as reinforcers. Thus, bitter or salty food, rather than quinine or sodium chloride *per se*, might serve as a negative reinforcer. Indeed, we were informed that when using bitter food, larval olfactory learning might be detectable in an *en masse* assay using relatively long reinforcer exposure (F. Mery, personal communication). Interestingly, the majority of sensory neurons from the larval pharyngeal gustatory sensillae seem to be retained into adulthood (Gendre et al., in press), so that a similar argument regarding quinine *versus* bitter food might apply in adults as well (Le Bourg and Buecher, 2002; Mery and Kawecki, 2002).

Candidate neuronal substrates

In the following, we speculate on candidate cells for visual and gustatory input, on a localization of visual memory, and on the modulatory neurons to mediate reinforcement.

Concerning vision, the Bolwig's organ is a prime candidate as it houses all known larval photoreceptors (Busto et al., 1999; Hassan et al., 2000). Concerning gustatory input, both the non-dome sensilla of the dorsal organ, the terminal organ, and the ventral organ are candidates, as they are necessary for gustatory choice behavior (Heimbeck et al., 1999; Liu et al., 2003). However, these external gustatory organs might be specifically involved in regulating preference and food uptake ('should I stay here and eat this?'), whereas the internal, pharyngeal sensillae might be involved in determining the quality of the swallowed food ('should I ever eat this again?').

Concerning memory localization, it was found in adult *Drosophila* that both aversive (electric shock) and appetitive (sucrose) olfactory memories, specifically memories dependent on the type I adenylate cyclase, can be localized to the same set of neurons in the mushroom bodies (Schwaerzel et al., in press; Zars et al., 2000). It will be interesting to see whether appetitive visual memories in larvae are localized to the mushroom bodies as well. This seems unlikely, however, as in adults substantial attempts to implicate the mushroom bodies in simple forms of visual associative learning yielded negative results (Heisenberg, 2003); indeed, in the fly, and as far as we know in any other experimental system, no localization of a visual memory has been reported to date.

An investigation into the modulatory system(s) that mediate the reinforcing effect of FRU might be guided by the finding that octopamine, but not dopamine, is necessary for appetitive olfactory learning in adult flies (Schwaerzel et al., in press). That study follows on the analysis of honeybee olfactory learning that identified an octopaminergic neuron as sufficient to mediate the reinforcing effect of the sucrose reward (Hammer and Menzel, 1995). At least with respect to dopamine, Python and Stocker (2002b) described candidate neurons in the *Drosophila* larva, providing a starting point also for the analysis of this system.

Outlook

This study on visual learning in *Drosophila* larvae complements the one by Scherer et al. (2003) on olfactory learning. Together, they offer the possibility for a comparative analysis of the organization of visual and olfactory memories and their potential interaction. We hope that the cellular simplicity of the larval nervous system will be useful for such approaches. In addition, the technical simplicity of both paradigms (i.e. they do not require elaborate equipment or technical skill) will hopefully make them easy to implement in other laboratories. Finally, both learning paradigms use individually assayed larvae; this will hopefully contribute towards more closely linking behavioral analysis and physiology. This seems desirable, particularly to relate behavioral and synaptic plasticity, as the former has so far been largely analyzed in adults and the latter in larvae.

The VolkswagenFoundation is acknowledged for generously providing the start-up funds for this research program (grant I/76 240 to B.G.). Current financial support comes from the Deutsche Forschungsgemeinschaft (SFB 554, to M.H.) and the Swiss National Fund (31-063447.00, 31-52639.97, to R.F.S.). Our cordial thanks to S. Dambacher, G. Gramlich, R. Kethers and T. Völler for help with the experiments; G. Krohne for help with the SEM; W. Spatz for help with the SEM pictures; Hubert Gachoud for help with Fig. 1E; H. Kaderschabek for workshop support; the members of the Würzburg and Fribourg labs, especially T. Zars and G. Heimbeck, for encouragement and continuous critical contributions. The remarks of T. Zars and H. Tanimoto on earlier versions of this manuscript are gratefully acknowledged.

References

Aceves-Piña, E. O. and Quinn, W. G. (1979). Learning in normal and mutant *Drosophila* larvae. *Science* **206**, 93-96.

Borst, A. (1983). Computation of olfactory signals in *Drosophila melanogaster*. *J. Comp. Physiol. A* **152**, 373-383.

Brand, A. H. and Perrimon, N. (1993). Targeted gene expression as a means of altering cell fates and generating dominant phenotypes. *Development* **118**, 401-415.

Busto, M., Iyengar, B. and Campos, A. R. (1999). Genetic dissection of behavior: modulation of locomotion by light in the *Drosophila melanogaster* larva requires genetically distinct visual system functions. *J. Neurosci.* **19**, 3337-3344.

Dukas, R. (1998). Ecological relevance of associative learning in fruit fly larvae. *Behav. Ecol. Sociobiol.* **19**, 195-200.

Fiala, A., Spall, T., Diegelmann, S., Eisermann, B., Sachse, S., Devaud, J.-M., Buchner, E. and Galizia, C. G. (2002). Visualization of olfactory information in projection neurons using genetically expressed cameleon in *Drosophila melanogaster*. *Curr. Biol.* **12**, 1877-1844.

Forbes, B. (1993). Larval learning and memory in *Drosophila melanogaster*. Diploma Thesis, University of Würzburg, Germany.

Hammer, M. and Menzel, R. (1995). Learning and memory in the honeybee. *J. Neurosci*. **15**, 1617-1630.

Hassan, J., Busto, M., Iyengar, B. and Campos, A. R. (2000). Behavioral characterization and genetic analysis of the *Drosophila melanogaster* larval response to light as revealed by a novel individual assay. *Behav. Genet.* **30**, 59-69.

Hendel, T. (2003). Appetitives aber kein aversives olfaktorisches Lernen bei *Drosophila* Larven. Diploma Thesis, University of Würzburg, Germany.

Heimbeck, G., Bugnon, V., Gendre, N., Häberlin, C. and Stocker, R. F. (1999). Smell and taste perception in *Drosophila melanogaster* larva: Toxin expression studies in chemosensory neurons. *J. Neurosci.* **19**, 6599-6609.

Heisenberg, M. (2003). Mushroom body memoire- from maps to models. *Nat. Rev. Neurosci.* **4**, 266-275.

Heisenberg, M., Borst, A., Wagner, S. and Byers, D. (1985). *Drosophila* mushroom body mutants are deficient in olfactory learning. *J. Neurogenet.* **2**, 1-30.

Joiner, M. A. and Griffith, L. C. (1999). Mapping of the anatomical circuit of CaM kinase-dependent courtship conditioning in *Drosophila*. *Learn. Mem.* **6**, 177-192.

Kitamoto, T. (2001). Conditional modification of behavior in *Drosophila* by targeted expression of a temperature-sensitive shibire allele in defined neurons. *J. Neurobiol.* **47**, 81-92.

Koh, Y. H., Gramates, L. S. and Budnik, V. (2000). *Drosophila* larval neuromuscular junction: Molecular components underlying synaptic plasticity. *Microsc. Res. Tech.* **49**, 14-25.

Le Bourg, E. and Buecher, C. (2002). Learned suppression of photopositve tendencies in *Drosophila melanogaster*. *Anim. Learn. Behav.* **30**, 330-341.

Lee, T. and Luo, L. (2001). Mosaic analysis with a repressible cell marker (MARCM) for *Drosophila* neural development. *Trends Neurosci.* **24**, 251-254.

Liu, L., Leonard, A. S., Motto, D. G., Feller, M. A., Price, M. P., Johnson, W. A. and Welsh, M. J. (2003a). Contribution of Drosophila DEG/ ENaC genes to salt taste. *Neuron* **39**, 133-146.

Liu, L., Yermolaieva, O., Johnson, W. A., Abboud, F. M. and Welsh, M. J. (2003b). Identification and function of thermosensory neurons in *Drosophila* larvae. *Nat. Neurosci.* **6**, 267-273.

Mery, F. and Kawecki, T. (2002). Experimental evolution of learning ability in fruit flies. *Proc. Natl. Acad. Sci. USA* **99**, 14274-14279.

Phelps, C. B. and Brand, A. H. (1998). Ectopic gene expression in *Drosophila* using GAL4 system. *Methods: A Companion to Methods in Enzymol.* **14**, 367-379.

Python, F. and Stocker, R. F. (2002a). Adult-like complexity of the larval antennal lobe of *D. melanogaster* despite markedly low numbers of odorant receptor neurons. *J. Comp. Neurol.* **445**, 374-387.

Python, F. and Stocker, R. F. (2002b). Immunoreactivity against choline acetyltransferase, γ-aminobutyric acid, histamine, octopamine, and serotonin in the larval chemosensory system of *Drosophila melanogaster*. *J. Comp. Neurol.* **453**, 157-167.

Rubin, G. M., Yandell, M. D., Wortman, J. R., Gabor Miklos, G. L., Nelson, C. R., Hariharan, I. K., Fortini, M. E., Li, P. W., Apweiler, R., Fleischmann, W. et al. (2000). Comparative genomics of the eukaryotes. *Science* **287**, 2204-2215.

Sawin-McCormack, E. P., Sokolowski, M. B. and Campos, A. R. (1995). Characterization and genetic analysis of *Drosophila melanogaster* photobehavior during larval development. *J. Neurogenet.* **10**, 119-135.

Scherer, S. (2002). Associative olfactory learning in individually assayed *Drosophila* larvae. Diploma Thesis, University of Fribourg, Switzerland.

Scherer, S., Stocker, R. F., and Gerber, B. (2003). Olfactory learning in individually assayed *Drosophila* larvae. *Learn. Mem.* **10**, 217-225.

Schmucker, D., Su, A. L., Beerman, B., Jackle, H. and Jay, D. G. (1994). Chromophore-assisted laser inactivation of patched protein switches cell fate in the larval visual system of *Drosophila*. *Proc. Natl. Acad. Sci. USA* **91**, 2666-2668.

Schwaerzel, M., Monastiroti, M., Scholz, H., Friggi-Grelin, F., Birman, S. and Heisenberg, M. (in press). Dopamine and octopamine differentiate between aversive and appetitive olfactory memories in *Drosophila*. *J. Neurosci.*, in press.

Scott, K., Brady, R. J., Cravchik, A., Morozov, P., Rzhetsky, A., Zuker, C. and Axel, R. (2001). A chemosensory gene family encoding candidate gustatory and olfactory receptors in *Drosophila*. *Cell* **104**, 661-673.

Sokolowski, M. B. (2001). *Drosophila*: genetics meets behavior. *Nat. Rev. Genet.* **2**, 879-890.

Stocker, R. F. (1994). The organization of the chemosensory system in Drosophila melanogaster: a review. *Cell Tissue Res.* **275**, 3-26.

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. *Microsc. Res. Tech.* **55**, 284-296.

Tempel, B. L., Bonini, N., Dawson, D. R. and Quinn, W. G. (1983). Reward learning in normal and mutant *Drosophila*. *Proc. Natl. Acad. Sci. USA* **80**, 1482-1486.

Tully, T., Cambiazo, V. and Kruse, L. (1994). Memory through metamorphosis in normal and mutant *Drosophila*. *J. Neurosci.* **14**, 68-74.

Waddell, S. and Quinn, W. G. (2001). Flies, genes and learning. *Ann. Rev. Neurosci.* **24**, 1283-1309.

Zars, T. (2000). Behavioral functions of the insect mushroom bodies. *Curr. Opin. Neurobiol.* **10**, 790-795.

Zars, T., Fischer, M., Schulz, R. and Heisenberg, M. (2000). Localization of a short-term memory in *Drosophila*. *Science* **288**, 672-675.