Odour avoidance learning in the larva of *Drosophila melanogaster*

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Drosophila larvae can be trained to avoid odours associated with electric shock. We describe here, an improved method of aversive conditioning and a procedure for decomposing learning retention curve that enables us to do a quantitative analysis of memory phases, short term (STM), middle term (MTM) and long term (LTM) as a function of training cycles. The same method of analysis when applied to learning mutants *dunce*, *amnesiac*, *rutabaga* and *radish* reveals memory deficits characteristic of the mutant strains.

1. Introduction

Quinn, Harris and Benzer first described electroshock conditioning in *Drosophila melanogaster* (Quinn *et al*. 1974). For more than thirty-five years, this paradigm has been widely employed in many laboratories to study genetics and molecular basis of associative conditioning. See reviews by (Dubnau and Tully 1998; Roman and Davis 2001; Waddell and Quinn 2001; Davis 2005). Aceves-Pina and Quinn also described aversive learning with electric shock in normal and mutant larvae and Tully *et al*. reported the passage of olfactory memory from larvae to imago through metamorphosis (Aceves-Pina and Quinn 1979; Tully *et al*. 1994). But, on the whole, learning in larvae has not received as much attention as learning in imago. Recently, there has been a revival of interest in larval learning, reviewed by Gerber and Stocker (2007). Recent experiments with larvae have employed appetitive reinforcement with sugar as reward and quinine or salt as punishments (Scherer *et al*. 2003; Hendel *et al*. 2005; Honjo and Furukubo-Tokunaga 2005). Gerber and his associates have studied olfactory conditioning in singly assayed larvae in a two-odour reciprocal paradigm. To one of the groups, odour A is presented with gustatory reward and odour B without reward (A⁺/B); the reciprocal group received A without reinforcement and B with reward (A/B⁺). The results establish Pavlovian associativity of learning.

So far, learning scores in larval conditioning experiments have been modest and their variance, high. They do not permit a detailed analysis of the stability of larval olfactory memory. In electroshock conditioning experiments with the imago, learning scores reaching 0.9 on a scale of 0 to 1 can be obtained, making possible a quantitative analysis of retention curves (de Belle and Heisenberg 1994). In this paper, we describe an improved procedure for electroshock conditioning of larvae, which gives learning scores comparable to the imago. We have decomposed the polyphasic learning retention curve of larvae into its components and examined the effect of repetition of training on the short term and relatively long term memory. Electroshock memory in the imago has been analyzed by examining the effect of gene mutations, anesthetics and treatments that interfere with protein synthesis. At least four subdivisions of memory have been recognized. Mutations in *rutabaga* and *dunce* affect anesthesia-sensitive short term memory (STM); *radish* eliminates middle term memory (MTM) not requiring protein synthesis while *amnesiac* affects long term memory (LTM) (Davis 2005; Waddell and Quinn 2001). In this paper, we decompose the memory curves from the four mutant strains and measure

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the formation and decline of different components of larval learning retention curve.

2. Materials and methods

2.1 *Stocks and cultures*

Experiments were carried out with the strain CsBz of *Drosophila melanogaster* and its mutants *dunce^M11*, *rutabaga^2080*, *radish^1* and *amnesiac*. Cultures were maintained on standard cornmeal medium (Lewis 1960). For producing larvae, 100 flies (50 ♂ and 50 ♀) were allowed to lay eggs on yeasted cornmeal for 12 h in bottles that were incubated at 25ºC for 4 days under a light/dark cycle of 12 h.

The larval ringer solution is made of 128 mM NaCl, 4.7 mM KCl, 1.8 mM $CaCl_2$, 0.9 mM Na_2HPO_4 and 0.37 mM KH_2PO_4 (Robb 1969).

Odorants were prepared by diluting stock chemicals of high purity (99+% from Sigma Aldrich Chemicals) in odour free liquid paraffin.

2.2 *Electroshock training*

Third instar larvae of 96-102 h old were separated by floating on 30% polyethylene glycol and washed in running tap water. About 400 larvae were placed at the centre of a 9 cm petri dish containing 10 ml of 20 mM lithium chloride solidified with 1.5% agarose. The larvae are confined within a ring made of a 4.5 cm petri dish whose bottom has been replaced by a plastic mesh. 0.5 ml of LiCl was spread on the inner surface of the petri dish. This prevents drying and ensures uniform passage of current. There is no LiCl in the medium on the test plate. This inner ring was covered with a lid on which 20 μl of appropriately diluted odorant was dispensed. The odour-bearing lid can be replaced quickly between training sessions. The larvae are placed in the ring and exposed to the odorant by covering with the lid for 30 s before being shocked for 30 s by passing AC current across a voltage gradient of 14 V/cm between the electrodes (figure 1).

2.3 *Larval response*

In experiments with single chemicals, olfactory response was measured in plate tests similar to those described by Heimbeck (Heimbeck *et al*. 1999; Kinsey 2001). Two symmetrically opposite zone were demarcated by arcs of radius 2 cm, drawn from the periphery of a 9 cm petri dish containing 10 ml of ringer solidified with 1% agar; 20 μl of the same odorant was spotted on blotting paper discs near the edge in both odour zones. About 50 larvae were placed
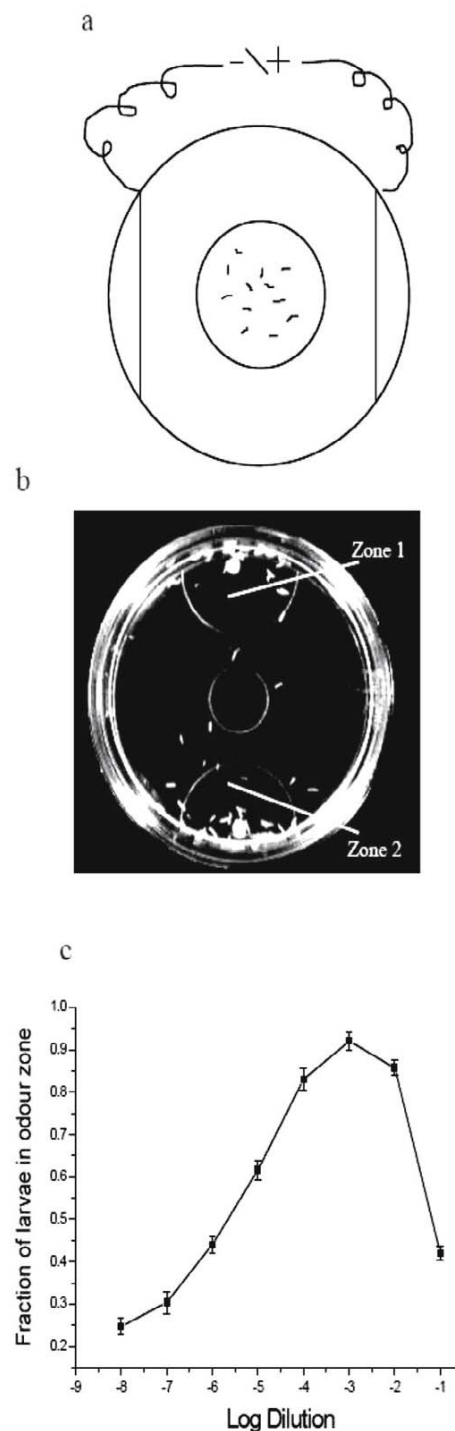

Figure 1. Aversive conditioning and measurement of response. (**a**) Sketch of arrangement for electroshock conditioning; the central dish is covered with the odour-bearing lid. (**b**) Larval plate test; $RI = \dfrac{\text{Number in Zone 1 + Number in Zone 2}}{\text{Total}}$. (**c**) Larval response to ethyl acetate. Attraction reaches a maximum at 10^{-3} after which aversion set in.

at the centre and the dish was covered. Larval counts were made from photographic records taken at 2 min.

When the odorant is attractive, the larvae move to the odour spot; when the odorant is aversive, they avoid the odour zone (figure 1). Attraction is measured by the fraction of larvae in the odour zones, i.e.

$$\text{Attraction Index} = \frac{\text{Number in Zone 1} + \text{Number in Zone 2}}{\text{Total (Zone 1} + \text{Zone 2} + \text{larvae outside both zones)}} \tag{1}$$

The attraction index is the probability P(O) of being in either of the odour zones corrected for the background (i.e. the probability of being in the marked zone in the absence of odour).

Learning is measured by the loss of attraction due to training i.e. association of odour with shock.

$$\text{Learning Index LI} = \frac{\text{Att. index}_{\text{control}} - \text{Att. index}_{\text{trained}}}{\text{Att. index}_{\text{control}}}. \tag{2}$$

Attraction to chemicals used by us such as ethyl acetate reaches its maximum at a dilution of around 10^{-3} when aversion set in. In order to observe increase in aversion (i.e. loss of attraction), we have tested the larvae just after the threshold of aversion (i.e. 10^{-2}). The controls are untrained larvae exposed to neither odour nor electric shock. Shock or exposure to odour by themselves, do not effect the response.

2.4 *Preference learning*

In this mode of training, the larvae were given electric shock in the presence of either of two odorant A and B. The trained larvae were tested in the presence of both odorants, one in each of the odour zone, O_A and O_B. The concentration of the two odorants are so adjusted that they are equally attractive (near zero preference). After aversive conditioning, the larvae avoid the odour associated with electric shock and go to the opposite odour zone (figure 4). Preference index, PI is defined as:

$$\text{Preference A over B PI}_{A/B} = \frac{\text{Number in Zone A} - \text{Number in Zone B}}{\text{Total (Zone 1} + \text{Zone 2} + \text{larva outside both zones)}} \tag{3}$$

$$\text{Preference B over A PI}_{B/A} = \frac{\text{Number in Zone B} - \text{Number in Zone A}}{\text{Total (Zone 1} + \text{Zone 2} + \text{larva outside both zones)}} \tag{4}$$

Preference learning is measured by change in preference. Learning index is defined as the average preference change for the two odorants measured independently.

$$\text{Preference Learning Index PLI} = \frac{P_{A/B} + P_{B/A}}{2} \tag{5}$$

The above is analogous to the learning index used by Tully and Quinn (Tully *et al.* 1994; Tully and Quinn 1985)

Table 1. Single chemical olfactory avoidance learning (OAL)

Untrained				
Plate No.	Larvae in zone 1	Larvae in zone 2	Outside 1 and 2	Total
1	23	22	7	52
2	25	22	3	50
3	21	24	6	51
4	19	23	6	48
Total	88	91	22	201
Trained (EA + Shock)				
Plate No.	Larvae in zone 1	Larvae in zone 2	Outside 1 and 2	Total
1	6	3	38	47
2	3	5	45	53
3	5	3	43	51
4	4	6	39	49
Total	18	17	165	200

Att. I $_{\text{Untrained}}$ = 0.89, Att. I $_{\text{trained}}$ = 0.175.
Learning Index LI = 0.80.

and represents associative conditioning independent of the two odours.

2.5 *Statistics*

In experiments on kinetics of memory decay, each time point is based on 200 larvae distributed equally on 4 petri dishes. Representative experiments are presented in tables 1 and 2. The curves subjected to Scatchard analysis (figure 6) are averages of 10 such experiments. Error bars show standard deviation of the mean (sdm). Significance of differences was estimated by *t*-test after checking normality by ANOVA.

3. Results

Electroshock conditioning with *Drosophila* larvae reported by Aceves-Pina and Quinn (Aceves-Pina and Quinn 1979) was done with odorants amyl acetate and 3-octanol. They obtained a learning index of 0.26 ± 0.02 from a mixed population of males and females and 0.21 ± 0.02 with third instar female larvae. The learning scores reported by other experimenters have also been in the same range i.e. between 0.2 and 0.3. With learning scores of this magnitude, it is not easy to carry out a detailed analysis of learning retention. We therefore, made an attempt to increase the efficiency of aversive conditioning by experimenting with other chemicals and by optimizing training and testing procedures. After preliminary experiments with a number of

Table 2. Preference learning. Ethyl acetate vs. isoamyl acetate

	Untrained			
Plate No.	Zone in A (EA)	Zone in B (IAA)	Outside A and B	Total
1	21	25	3	49
2	24	22	2	48
3	29	21	4	54
4	19	27	5	51
Total	93	95	14	202
	EA + Shock			
Plate No.	Zone in A (EA)	Zone in B (IAA)	Outside A and B	Total
1	3	49	2	54
2	1	39	5	45
3	4	43	1	48
4	3	40	5	48
Total	11	171	13	195
	IAA + Shock			
Plate No.	Zone in A (EA)	Zone B (IAA)	Outside A and B	Total
1	42	2	5	49
2	37	5	1	43
3	43	2	3	48
4	45	3	7	55
Total	167	12	16	195

$PI_{Untrained} = 0.09$, $PI_{Trained}$ A/B = 0.82, $PI_{Trained}$ B/A = 0.79.
Learning Index PLI = 0.78.

chemicals, the following six chemicals; ethyl acetate (EA), isoamyl acetate (IAA), isobutyl acetate (IBA), ethyl butyrate (EB), butyl butyrate (BB), 1-butanol (But) and 1-hexanol (Hex), were chosen. All these chemicals are attractants at low concentration and become aversive at dilution less than 10^{-3}.

3.1 *Olfactory avoidance learning with single chemicals*

Third instar larvae were trained to avoid ethyl acetate as described in methods and samples taken at various times after training were tested. About 200 larvae were distributed on four test plates and the numbers in three zones were counted at 2 min. The result of a representative experiment is presented in table 1. Subsequent graphs and tables are based on ten such experiments. Experiments with three different odorants ethyl acetate, isoamyl acetate and 1-butanol are summarized in figure 2.

Three different controls were employed (i) untreated larvae i.e. larvae not exposed to odour or electric shock,

(ii) larvae exposed to odour but not shock and (iii) larvae given electric shock in the absence of odorant. The short exposure of 1 min to any of the odorants does not affect the attraction index (i.e. 2, 3, and 4 in figure 2a). Association of shock with odour brought down attraction index from 0.85 to 0.15. Learning indices calculated from the fall in attraction are shown in figure 2b. These are 0.82, 0.84 and 0.87 respectively for EA, IAA and 1-butanol. The controls used in these calculations are untreated larvae i.e. not exposed to odour or electric shock.

3.2 *Associativity*

We have obtained additional evidence of associativity in single odour avoidance learning by varying the temporal relation between odour presentation and shock. The larvae were exposed to EA either before or after the 30 s electric shock. The result (figure 3) is similar to that obtained by Tanimoto *et al*. with the imago (Tanimoto *et al*. 2004). The electric shock is most effective when it just follows the odorant. Indeed, if the punishment is given before the odour cue, learning index changes from positive to negative. Tanimoto *et al*. ascribe this affect to learning of "relief" from shock.

3.3 *Dependence of learning on repetition and spacing*

Memory of olfactory learning in both larvae and imago persists for different periods depending upon the training regimen. Repetitive training with appropriate spacing between training cycles can produce memories which, in the imago, may last for several days (Quinn and Dudai 1976; Aceves-Pina and Quinn 1979; Tully and Quinn 1985). The effect of repetition and spacing between training cycles in our protocol is shown in figure 4. The learning index increased with the number of training cycles reaching a plateau in 10 cycles. A rest interval of 5 min is optimal for learning. Both repetition and inter-training interval affect the stability of memory. This is discussed in sections 3.5 and 3.6, which deal with effect of repetition on the learning retention curve.

3.4 *Olfactory preference learning with two chemicals*

As Tully *et al*. (1994) point out, learning experiments with a single odorant as cue and shock as negative reinforcement are not sufficient. For a convincing demonstration of associative learning, it is better to employ two odorants and show a transfer of response from one to the other. This can be done in preference learning experiments.

Third instar larvae of 96-102 h old were given electric shocks in the presence of one or the other of a pair of
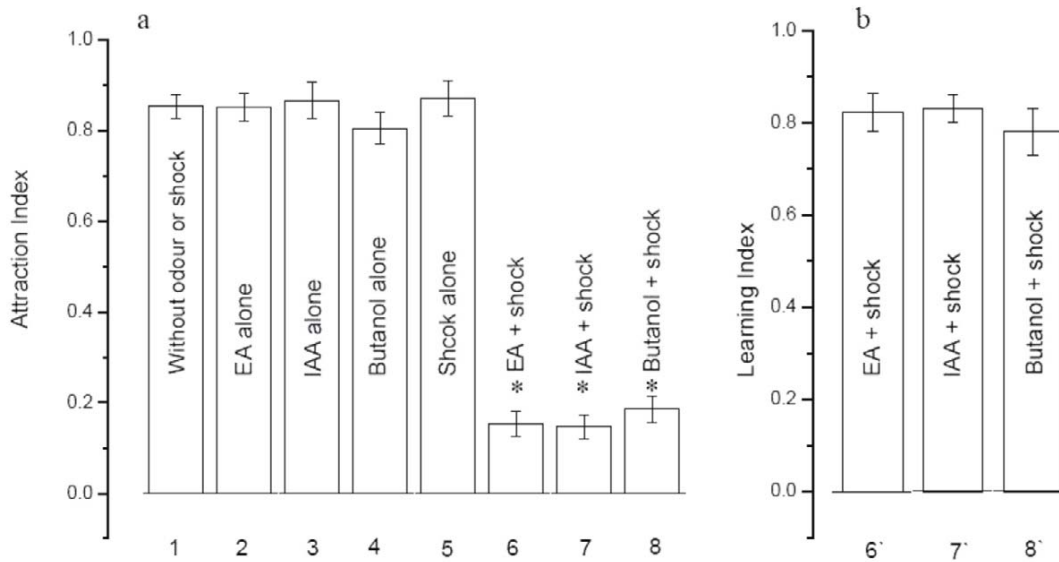
Figure 2. Olfactory avoidance learning (OAL) in larvae. (**a**) 1-5 controls; 6-8 effect of pairing shock with three different odours. The differences are significant between treated and untreated (* $P<0.0001$). (**b**) 6`-8` learning indices calculated from changes in RI. The differences between odours are not significant. Error bars indicate sdm. $N=10$ experiments for each chemicals.
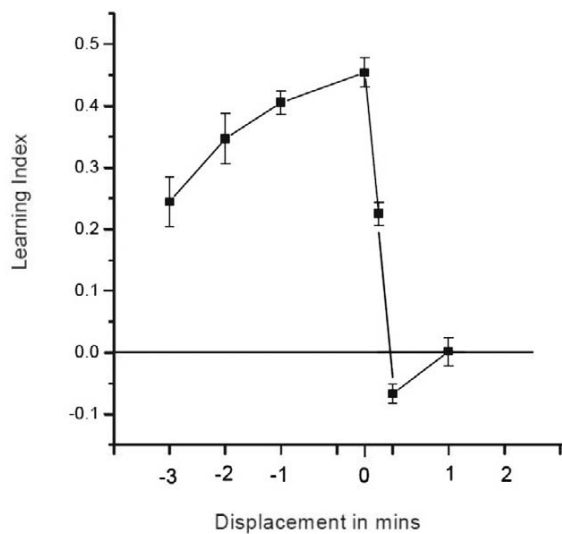

Figure 3. Effect of temporal displacement of odour (EA 10^{-2}) from shock. Zero on the displacement scale represents the mid point of shock. N= 10 for each time point. Error bars represent sdm.

odorants (A or B) as described in methods and tested on plates with equally attractive amounts of the odorants placed in the two odour zones O_A and O_B on opposite sides of the petri dish. Two preference indices could be measured as defined earlier in 2.4.

Results of a representative experiment with ethyl acetate and isoamyl acetate are given in table 2, which exemplifies the relevant calculations. Experiments with four different pairs of odorants are summarized in figure 5. Preference learning is odour-independent. All pairs of odorants tested gave equally high learning indices similar to learning indices in experiments with single chemicals.

3.5 *Memory of electroshock conditioning*

The simplicity of the training paradigm and the high learning scores obtained enable us to do kinetic analysis of memory decay. This analysis has been carried out in single odour learning experiments with EA described in section 3.1. Learning retention curves after 1, 3, 5 and 10 cycles of training are shown in figure 6. Data from 2, 4, 6, 7, 8 and 12 cycles (*see* figure 4) have been omitted for clarity.

Larvae were trained by pairing EA with shock with a 5 min spacing between training cycles. The initial learning index was measured 1 min after the last cycle and the retention curve was followed for 8 h. Learning index rose from 0.27 after a single cycle to 0.82 after 10 cycles of training.

The memory curves are polyphasic and exhibit a rapidly declining and a slowly declining phase. The relative proportions of different phases changed with the number of training cycles and with time. In order to analyze the memory curve, it is necessary to separate the phases and to follow their appearance and decay.
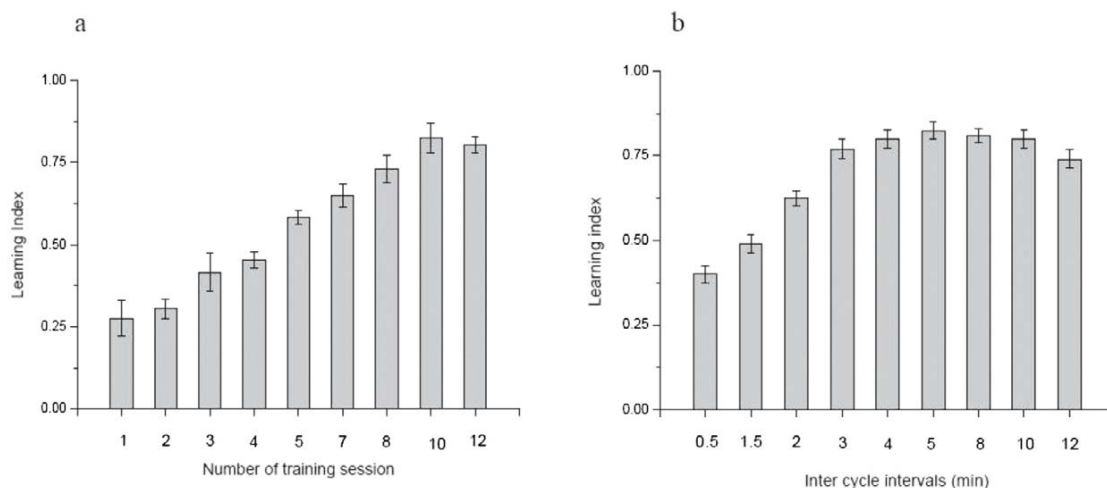
Figure 4. Dependence of odour avoidance learning on (**a**) number of training cycles and (**b**) spacing between cycles. Error bars represent sdm of 10 experiments.
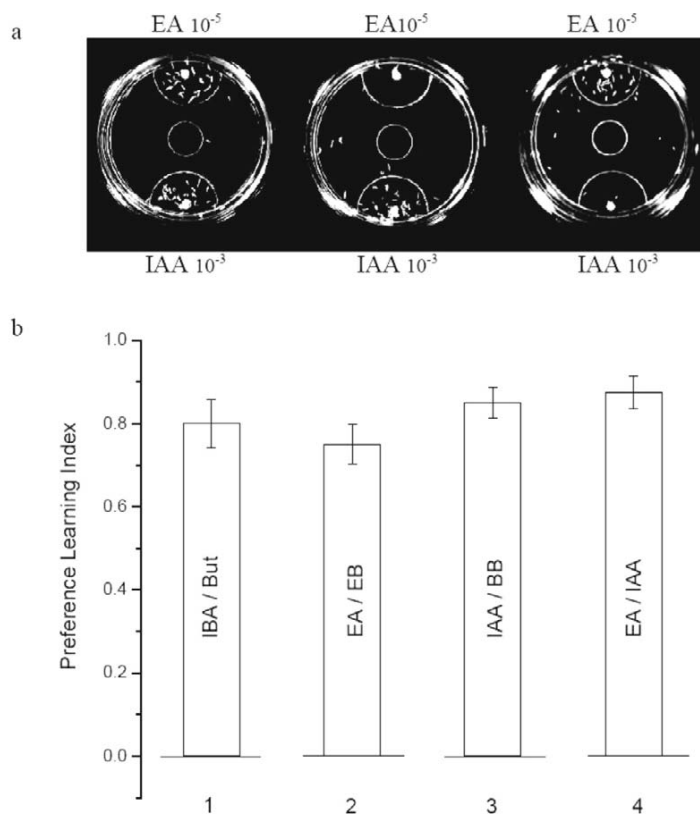

Figure 5. Preference learning. (**a**) Test plates showing preference test between two odorants EA / IAA. The dish on the left shows untrained larvae. 10^{-5} EA and 10^{-3} IAA attract untrained larvae equally. Pairing odour with shock drives the larvae to the opposite zone. Preference learning index $\text{PLI} = \dfrac{P_{EA/IAA} + P_{IAA/EA}}{2}$. (**b**) Preference learning between four different combinations of odorants IAB (isoamyl butyrate), EA (ethyl acetate), EB (ethyl butyrate), IAA (isoamyl acetate) and BB (butyl butyrate). Error bars represent SEM of six experiments.
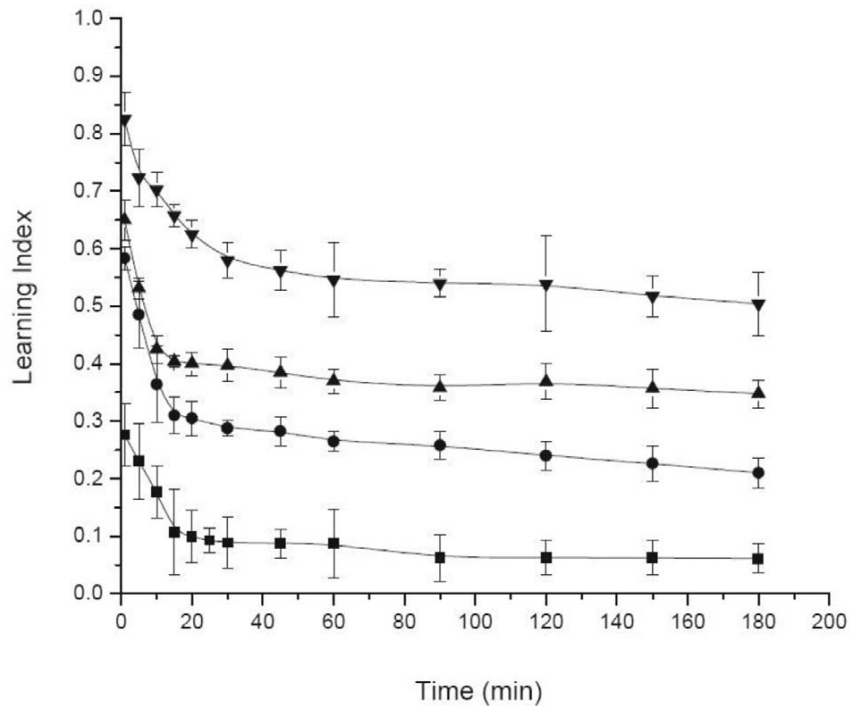
Figure 6.   Effect of repetition of training on learning retention. Number of training cycle ■ 1, ● 3, ▲ 5 and ▼ 10.

3.6   *Decomposition of learning retention curves*

To decompose the memory curve, we have employed a method, involving successive stripping of the curve introduced in kinetic analysis by Scatchard (Scatchard 1949; Klotz and Hunston 1971). The procedure consists of subtracting the most stable component from the total curve to obtain the rapidly decaying component. If the residual curve is still polyphasic, the step can be repeated until curves with uniform decay rates are obtained.

The stepwise dissection of memory curves is illustrated in figure 7 showing the decomposition after 5 cycles of training. The curve is normalized to a total learning index of 1 at $t=0$ and plotted on a semi log graph paper. The slowly declining component is extrapolated to obtain an initial value of 0.38 for LTM. The curve for LTM is subtracted from the total. This yields a biphasic residual curve containing STM and MTM. MTM is now extrapolated to $t=0$ and subtracted from the residual to obtain STM. The procedure gives an initial value of 0.18 for MTM and 0.44 for STM. The results of Scatchard analysis of memory curve up to 8 cycles of training are given in figure 8 showing formation of STM, MTM and LTM and the decay curve for the three phases are shown in figure 9.

STM appears after the first cycle of training. Its amount and half life of 6-8 min do not seem to change appreciably

with training. LTM too is present after the first cycle of training, rising from 0.07 to 0.17 in the first four cycles and rapidly thereafter. Its half life remains unchanged at 1400 min. The intermediate phase, MTM is conspicuous by its absence after the first two cycles. It makes its appearance after the third cycle and saturates at 0.13. The half life of MTM on the other hand changes dramatically from 24 min after 4 cycles to 165 min after 5 cycles and about 600 min after 8 cycles.

3.7   *Effect of mutations*

One might ask whether, the three phases of the learning retention curve described in the previous section are merely the outcome of algebraic decomposition of a single curve or reflect a substantive underlying process. We have attempted to examine this issue by doing Scatchard analysis of memory curves from learning mutants.

Learning mutants whose memory curves have been carefully investigated by several groups are *rutabaga*, *dunce*, *amnesia* and *radish*. These mutants were discovered by Quinn and his collaborators and affect both imago and larvae (Dudai *et al.* 1976; Aceves-Pina and Quinn 1979; Quinn *et al.* 1979; Duerr and Quinn 1982; Livingstone
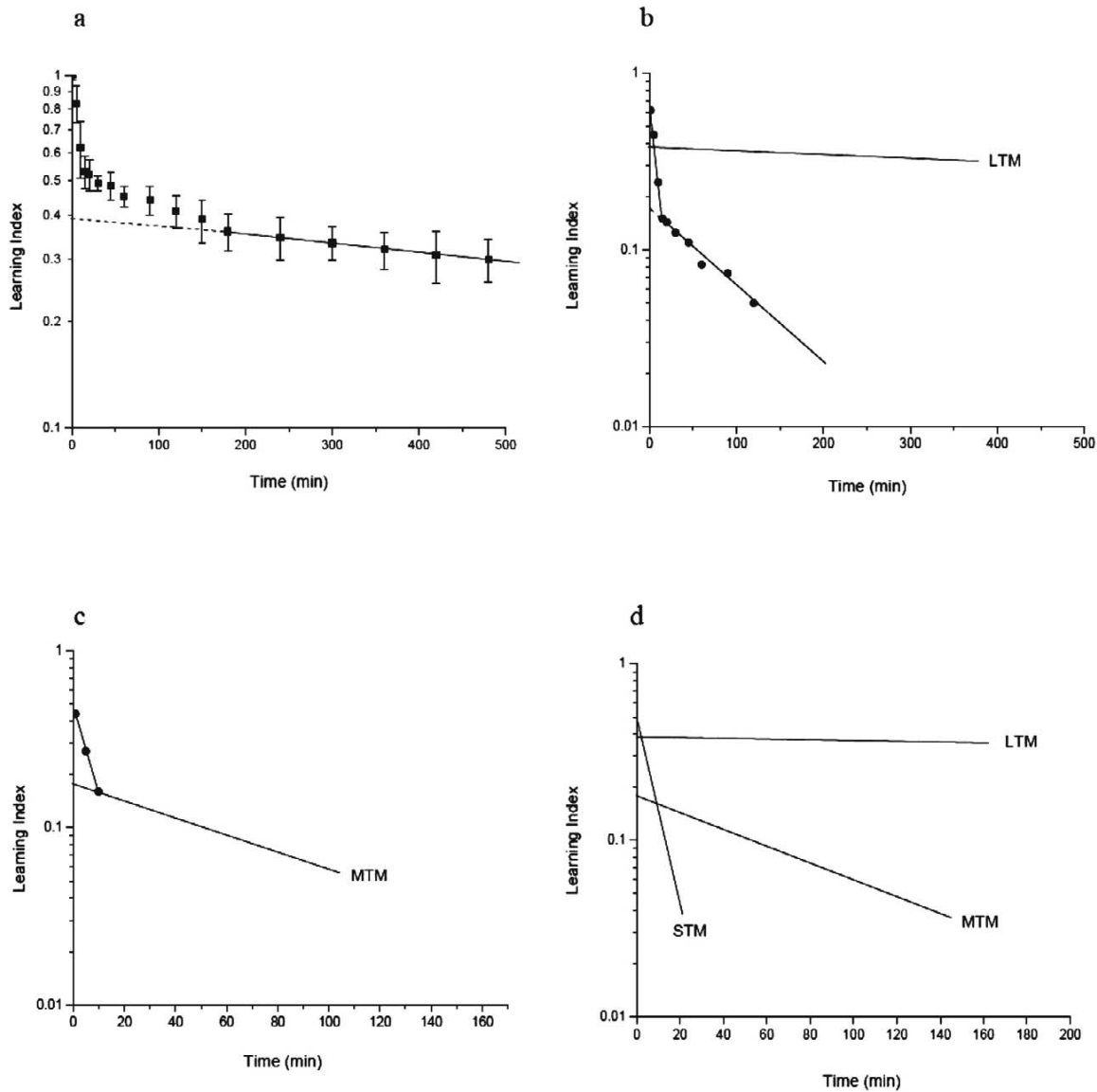
Figure 7. Step-wise decomposition of learning curve after 5 cycles. (**a**) curve normalized. (**b**) LTM subtracted leaving a biphasic residual curve. (**c**) MTM subtracted. (**d**) separated phases, STM, MTM and LTM.

et al. 1984; Folkers et al. 1993). The mutants have been used to dissect the phases of memory in the imago (Dubnau and Tully 1998; Waddell and Quinn 2001). *Dunce* and *rutabaga* affect short term memory; *amnesiac* affects long term memory and *radish* affects middle term memory. In combination with anesthetics and inhibitors of protein synthesis, the mutants serve to define four parts of olfactory memory in *Drosophila*, Short term STM, middle term MTM and two types of long term memory LTM, anesthesia sensitive LTM and anesthesia resistant LLTM (Dubnau and Tully 1998; Waddell and Quinn 2001).

We decomposed the learning retention curves from mutants' *rut*, *dnc*, *radish* and *amn* and compared them with the wild-type. The result of this analysis shows that the memory deficit in each case after Scatchard decomposition is as expected; *rut* and *dnc* lack STM while *radish* lacks MTM. The long term memory in *amn* is not completely abolished but reduced to less than half after 60 min of decay (figure 10).
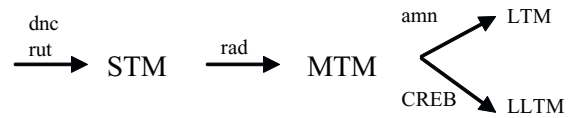
4. Discussion

The method of decomposition of learning curve employed by us is a simple graphic procedure. It makes use of the fact that the phases of the memory curve become linear in a semi log plot. STM and LTM are linear to begin with. The residual intermediate phase, MTM also linearizes when LTM and STM are removed. The key observation is that the half lives of STM and LTM do not change during the 8 h

Figure 8. Development of three phases of memory curve as a function of training cycles. ■ STM, ● MTM and ▲ LTM.

that memory have been followed. MTM alone appears to be undergoing consolidation with repetition of training cycles. Whereas STM and MTM saturate in a few cycles of training, LTM continues to increase.

Conventionally, phases of memory have been considered as sequentially related, STM being a precursor of MTM and LTM. Evidence from several organisms shows that long term memory forms in parallel with other phases (Allweis 1991). In our experiments, STM and LTM are present after the first cycle of training, but MTM appears only after the third cycle of training.

Electroshock memory in the adult flies has been subdivided on the basis of its sensitivity to anaesthetics, inhibitors of protein synthesis and effects of single gene mutation into 4 types, STM, MTM, LTM and long lasting long term memory, LLTM (Dubnau and Tully 1998; Waddell and Quinn 2001; Davis 2005). Memory phases been arranged in a formal pathway

Are the phases of larval memory obtain by Scatchard decomposition the same as found in adult flies? The results with the four canonical mutants suggest that this is so. We are currently investigating the effect of inhibitors on larval memory. The results so far agree with the findings from mutants (AbuBaker *et al*, unpublished results) and reinforce the reliability of the method of analysis used. On the other hand, one need not expect an exact correspondence between memory phases identified by genetic and
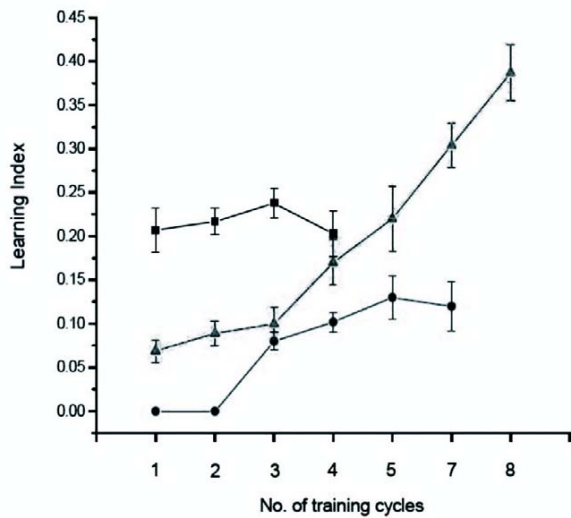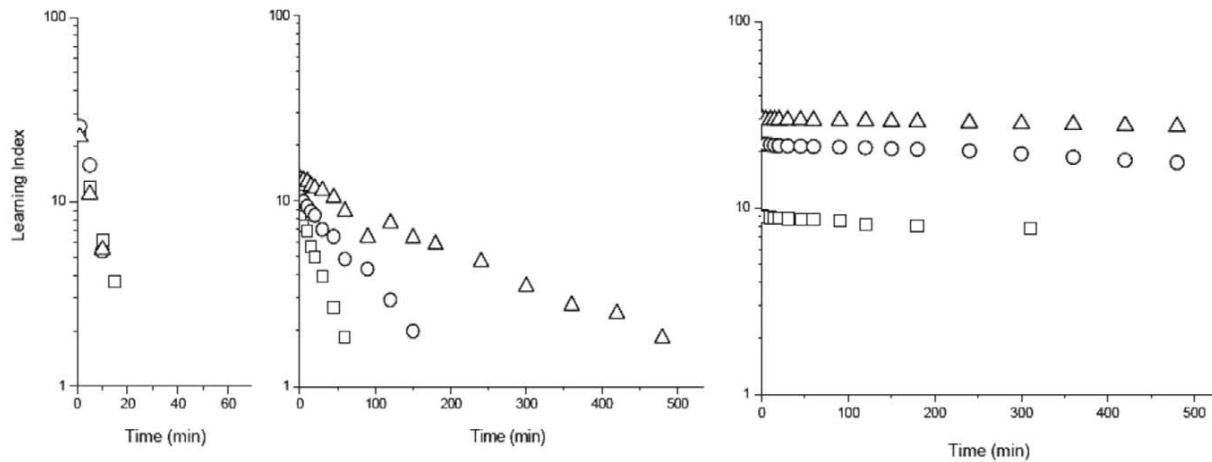

Figure 9. Decay rates of three phases of memory. Curve with increasing number of training cycles after Scatchard analysis. **(a)** STM; **(b)** MTM and **(c)** LTM. □ 3 cycles, ○ 5 cycles and ∆ 7 cycles.
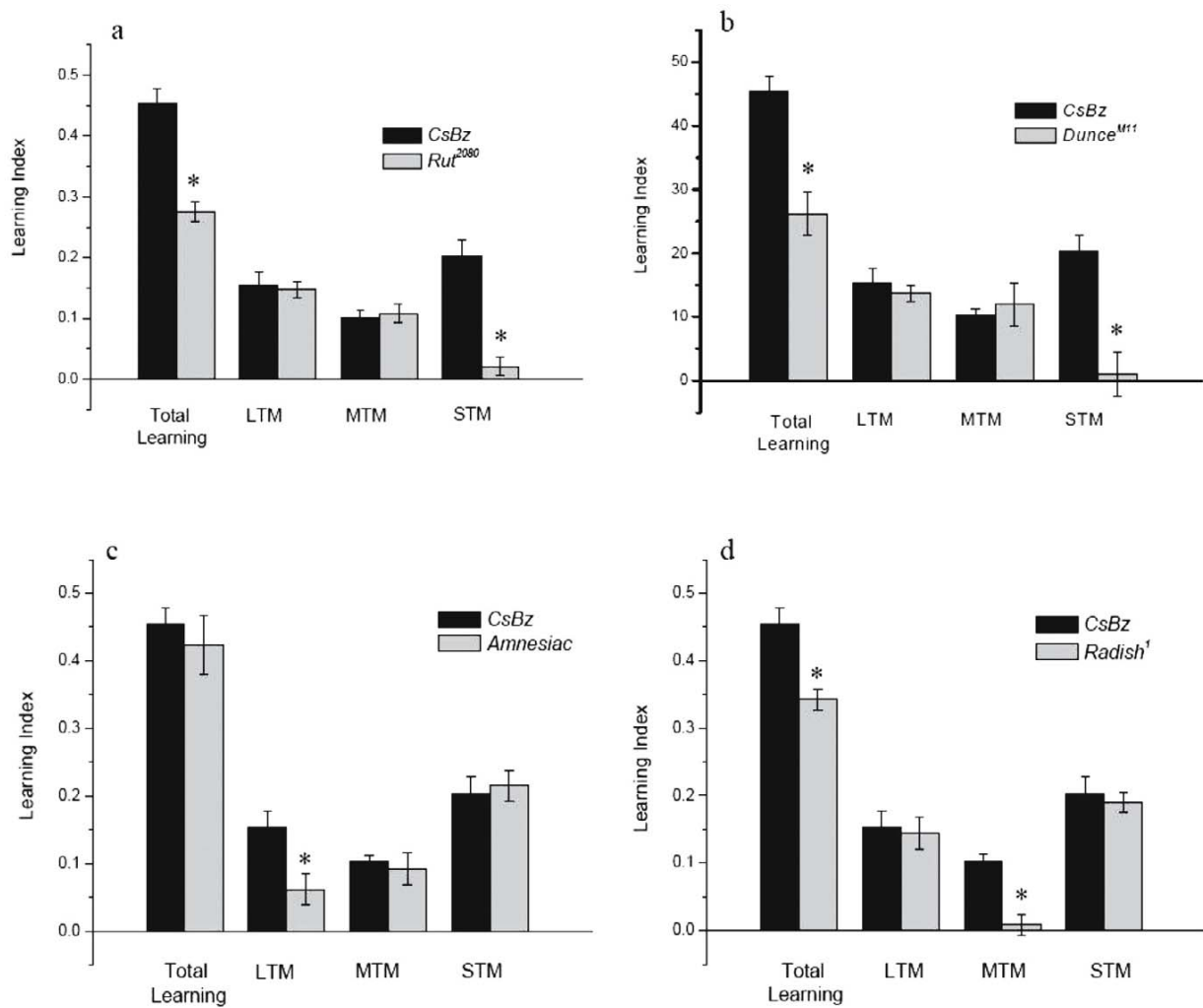
Figure 10. Effect of mutations on three phases of memory after 4 cycles of training. STM is blocked in *rut* and *dnc*. MTM is blocked in *radish*. *Amnesiac* has reduced LTM. The deficit in *amn* is not initially evident but is visible after 60 min. The error bars represent sdm. *N*=10. * *P*<0.0001.

biochemical criteria and behavioural experiments. It is very likely that the formation and decay of memory are influenced by age, developmental stage and conditions in which the animals are grown. This is a subject, which requires further study.

References

Aceves-Pina E O and Quinn W G 1979 Learning in normal and mutant *Drosophila* larvae; *Science* **206** 93–96

Allweis C 1991 The congruity of rat and chick multiphasic memory consolidation models; in *Neural and behavioral plasticity: the use of the domestic chick as a model* (ed.) R J Andrew. (New York: Oxford UP) pp 370–393

Davis R L 2005 Olfactory memory formation in *Drosophila*: from molecular to systems neuroscience; *Annu. Rev. Neurosci.* **28** 275–302

de Belle J S and Heisenberg M 1994 Associative odor learning in *Drosophila* abolished by chemical ablation of mushroom bodies; *Science* **263** 692–695

Dubnau J and Tully T 1998 Gene discovery in *Drosophila*: new insights for learning and memory; *Annu. Rev. Neurosci.* **21** 407–444

Dudai Y, Jan Y N, Byers D, Quinn W G and Benzer S 1976 dunce, a mutant of *Drosophila* deficient in learning; *Proc. Natl. Acad. Sci. USA* **73** 1684–1688

Duerr J S and Quinn W G 1982 Three *Drosophila* mutations that block associative learning also affect habituation and sensitization; *Proc. Natl. Acad. Sci. USA* **79** 3646–3650

Folkers E, Drain P and Quinn W G 1993 Radish, a *Drosophila* mutant deficient in consolidated memory; *Proc. Natl. Acad. Sci. USA* **90** 8123–8127

Gerber B and Stocker R F 2007 The *Drosophila* larva as a model for studying chemosensation and chemosensory learning: a review; *Chem. Senses.* **32** 65–89

Heimbeck G, Bugnon V, Gendre N, Haberlin 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

Hendel T, Michels B, Neuser K, Schipanski A, Kaun K, Sokolowski M B, Marohn F, Michel R, Heisenberg M and Gerber B 2005 The carrot, not the stick: appetitive rather than aversive gustatory stimuli support associative olfactory learning in individually assayed *Drosophila* larvae; *J. Comp. Physiol. A Neuroethol. Sens. Neural. Behav. Physiol.* **191** 265–279

Honjo K and Furukubo-Tokunaga K 2005 Induction of cAMP response element-binding protein-dependent medium-term memory by appetitive gustatory reinforcement in *Drosophila* larvae; *J. Neurosci.* **25** 7905–7913

Kinsey R H 2001 Olfactory learning in *Drosophila melanogaster* larvae; *Private Communication*

Klotz I M and Hunston D L 1971 Properties of graphical representations of multiple classes of binding sites; *Biochemistry* **10** 3065–3069

Lewis E B 1960 A new standard food medium; *Dros. Inf. Serv.* **34** 117–118

Livingstone M S, Sziber P P and Quinn W G 1984 Loss of calcium/calmodulin responsiveness in adenylate cyclase of rutabaga, a *Drosophila* learning mutant; *Cell* **37** 205–215

Quinn W G and Dudai Y 1976 Memory phases in *Drosophila*; *Nature (London)* **262** 576–577

Quinn W G, Harris W A and Benzer S 1974 Conditioned behavior in *Drosophila melanogaster*; *Proc. Natl. Acad. Sci. USA* **71** 708–712

Quinn W G, Sziber P P and Booker R 1979 The *Drosophila* memory mutant amnesiac; *Nature (London)* **277** 212–214

Robb J A 1969 Maintenance of imaginal discs of *Drosophila melanogaster* in chemically defined media; *J. Cell Biol.* **41** 876–885

Roman G. and Davis R L 2001 Molecular biology and anatomy of *Drosophila* olfactory associative learning; *Bioessays* **23** 571–581

Scatchard G 1949 The attractions of proteins for small molecules and ions; *Ann. N. Y. Acad. Sci.* **51** 660

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

Tanimoto H, Heisenberg M and Gerber B 2004 Experimental psychology: event timing turns punishment to reward; *Nature (London)* **430** 983

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

Tully T and Quinn W G 1985 Classical conditioning and retention in normal and mutant *Drosophila melanogaster*; *J. Comp. Physiol. A* **157** 263–277

Waddell S and Quinn W G 2001 Flies, genes, and learning; *Annu. Rev. Neurosci.* **24** 1283–1309