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# Aversive olfactory conditioning with NaCl induces middle-term memory in *Drosophila melanogaster* larvae

**Md. Zeeshan Ali, Anushree and Jawaid Ahsan**

*Drosophila* Behavioral Laboratory, Department of Biotechnology,
Central University of South Bihar, Gaya–824 236 (India)
*Corresponding author: Name: Jawaid Ahsan
Phone: +91- 8521627799
E-mail: **jahsan@cub.ac.in**

**Abstract**

The *Drosophila melanogaster*, commonly known as fruit fly, more correctly termed as the vinegar fly, has been extensively used as a powerful model organism for elucidation of various biological mechanisms in higher organisms including humans. To analyze associative learning, the olfactory classical conditioning using *D. melanogaster* larva is widely used in the field of behavioral neuroscience. The larva is simpler than adult with minimalistic neural network which make it an ideal model organism to elucidate the associative plasticity circuitry, physiology, and signaling. In this work, we have employed one robust training paradigm for associative olfactory conditioning. We describe the olfactory learning and memory formed using sodium chloride (NaCl) as the aversive unconditioned stimulus (US) and the odorant ethyl acetate (EA) as the conditioned stimulus (CS). Our results show that the association of EA/NaCl led to the formation of a middle-term memory (MTM) in fruit fly larvae. The learning acquired was strong and interestingly remained significant even after 3 hours of conditioning.

**Key words :** *Drosophila melanogaster*, larva, ethyl acetate, NaCl, learning and memory, middle-term memory.

Locating food, avoiding predators, and seeking suitable mates are essential for the survival of animals in the wild. Under such circumstances, associative learning and cognition become very critical for them. This capability of the brain allows animals to modify their present behavior with past experiences for increased chances of persistence[6,16]. To understand the underlying mechanism of associative learning, the olfactory classical conditioning using *Drosophila melanogaster* larvae are widely used in the field of behavioral

neuroscience. It involves the association of an odorant with an unconditioned stimulus (US). As a result of pairing, the odorant becomes a conditioned stimulus (CS), prompting a response similar to the US[1,8,14]. Also, the simple morphology and minimalistic neural network of fruit fly larva make it an appropriate model organism to decipher the associative plasticity circuitry, physiology, and signaling[7,8,12].

Previously it has been reported that the association of linalool (LIN) and pentyl acetate (PA) with NaCl results in a short-lived larval olfactory memory that decays within 20 minutes[8]. However, in the present study, we describe the association of ethyl acetate (EA), a robust larval attractant with NaCl. Unlike LIN and PA, the EA/NaCl olfactory conditioning induces a middle-term memory that remains strongly significant even 3 hours after conditioning.

*Fly stocks :*

The wild-type strain Oregon-R was used as the fly stock maintained at 25°C under a day/night cycle of 12 hours. Files were reared on standard corn meal media consisting of 8 gm/l agar (Himedia; 9002-18-0), 15 gm/l yeast extract, 80 gm/l corn, 20 gm/l, dextrose (Himedia; 50-99-7), and 40 gm/l, sucrose (Himedia; 57-50-1). Propionic acid, 4 ml/l (Himedia; 79-09-4), and ortho-phosphoric acid, 0.6 ml/l (Himedia; 7664-38-2), were added as fungicides.

*Chemicals & reinforcers :*

The chemicals used were obtained from Himedia, ThermoFisher, and G-Biosciences. The odorant ethyl acetate (EA) (141-78-6) was obtained from Sigma-Aldrich.

*Learning and memory experiments :*
*Temperature :*

The behavioral experiments on early third instar larvae were performed at 25 °C.

*Aversive olfactory conditioning with* NaCl:

For aversive olfactory conditioning with NaCl, freshly prepared 1% agar plates (90 mm glass Petri dishes) were used. In one agar plate, 1 ml of distilled water (DW) was spread as control on the surface of the solidified agar. In the second agar plate, 1 ml of 1M NaCl solution was spread as aversive stimulus. The early third-instar larvae were then placed on the training plate using a paintbrush. 10 µl of neat odorant (EA) was placed inside the lid on a filter disc. The lid was placed immediately on the training plate and left undisturbed for 30 minutes. Owing to this, the larvae experienced a simultaneous exposure to EA (CS) and NaCl (US) (Fig. 1). At the end of 30 minutes, the larvae were rinsed with DW in a plate to ensure that no residual odorant or NaCl remain stuck to their bodies. After the completion of training, the larvae were transferred to the testing plates. Larvae naive to both CS and US were used as an extra control.

*Quantification of the learning and memory formed :*

The larval plate assay, as per Khurana *et al*.,[10] was employed to measure the learning and memory formed after aversive olfactory classical conditioning using early third-instar larvae. For this, ~ 50 larvae were put in the

center of a 1% agar plate. On either side of the plate, there were two paper discs with 20 µl of EA ($10^{-2}$) diluted in mineral oil (Himedia; 8042-47-5) (Fig. 2). After 2 minutes, the number of larvae in different demarcated zones was counted to calculate the response index (RI).

$$\text{Response Index (RI)} = \frac{\text{Number of larvae in zone 1 } (O_1) + \text{Number of larvae in zone 2 } (O_2)}{\text{Total number of larvae } (O_1+O_2+C)}$$

*Statistics :*

To elucidate the statistical significance of the differences between conditioned and unconditioned response, Student's *t*-test and ANOVA were used. For additional confirmation, Mann-Whitney *U* test or the Kruskal-Wallis test were also performed. Error bars in the entire paper represent the standard deviation of the mean (SDM).

*Aversive olfactory conditioning with EA/*NaCl*:*

The early third-instar larvae were trained to associate EA with NaCl. After training, the larvae were tested using the larval plate assay to decipher the learning acquired. The association of EA/NaCl led to a significant decrement in the RI of the trained larvae in comparison to the controls signifying learning (Fig. 3).

*Memory decay curve of aversive olfactory conditioning with EA/*NaCl*:*

In aversive olfactory conditioning with EA/NaCl, there was a significant dip in the RI of the trained larvae compared to the control, *i.e.*, EA/DW. This decrease exhibited the formation of aversive memory. The memory formed gradually decayed with time but remained significant even 3 hours after conditioning (Fig. 4).

In the present study, we have characterized the larval aversive olfactory memory formed by associating EA with NaCl. Our results were in contradiction with an earlier work performed by Honjo & Furukubo-Tokunaga[8], using the same conditioning paradigm. In their case, they used the odorants linalool (LIN) and pentyl acetate (PA). They found that associating these odorants with 1M NaCl resulted in a short-lived memory that was lost within 20 minutes. However, in our study, we used the odorant ethyl acetate (EA), and its association with NaCl resulted in middle-term memory (MTM). The aversive MTM formed gradually decayed with time but was significant even 3 hours after conditioning.

Octopamine (OA) is known to facilitate sugar reward learning in *D. melanogaster*[3, 11,15]. In the fruit fly larvae, the octopaminergic (OA) neurons are intensively innervated with antennal lobe (AL) and mushroom body (MB) neurons. This hints towards the convergence of the CS (odorant) and the US (sucrose) signals at both the sites in appetitive olfactory conditioning resulting in a stable MTM[9]. However, it has been found that OA through the Octβ1R receptor also delivers the aversive US information to the α/β KCs in *D. melanogaster* [15]. So, the aversive olfactory conditioning with EA/NaCl might be activating the OA neuronal pathway, thereby relaying the CS (EA) and the US (NaCl) signals to both the AL and MB sites. Consequently, this results in the formation of the aversive MTM.

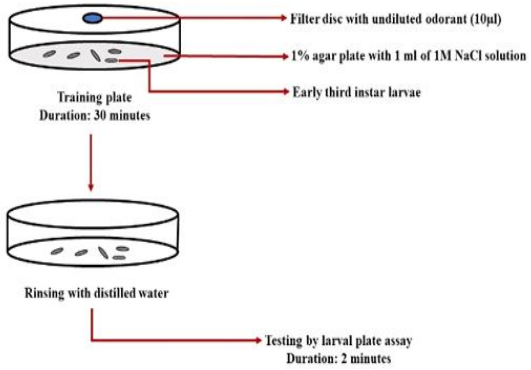


**Fig. 1.** Aversive olfactory conditioning with NaCl: Early third-instar larvae (3 days after egg laying) were put on 1% agar plates with distilled water (DW) (control) or 1 ml of 1 M NaCl solution (aversive US) spread on them. 10 µl of undiluted odorant (CS) on a filter disc was placed inside of the lid covering the plate. The setup was left untouched for 30 minutes.

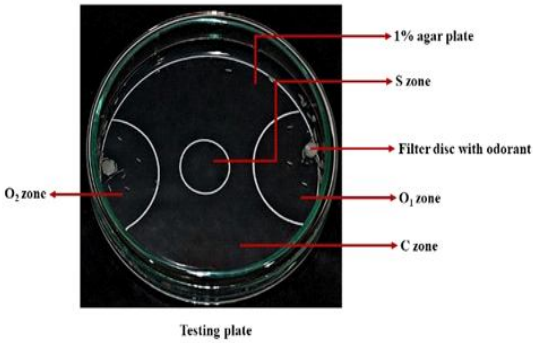


**Fig. 2.** Larval plate assay: S 50 larvae were put in the S zone. 20 µl of an odorant diluted in mineral oil was placed on the filter discs in the $O_1$ and $O_2$ zones each. After 2 minutes, the numbers of larvae in demarcated zones were counted, and RI was calculated.

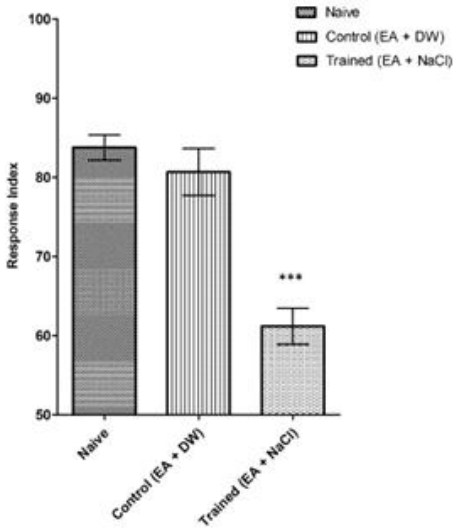


**Fig. 3.** Aversive olfactory conditioning in *D. melanogaster* larvae. Significant decrease in RI was observed when EA was associated with 1M NaCl. ***p <0.0001 with ANOVA compared with any of the two other conditions; also cross-checked by Kruskal-Wallis test, p <0.0001.

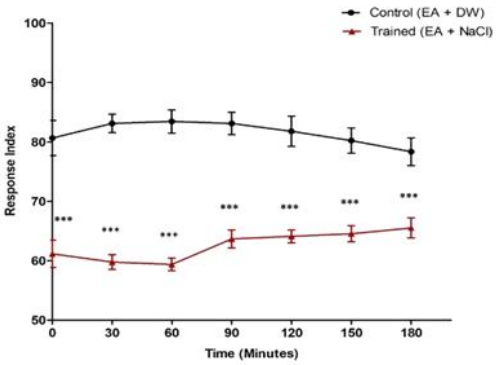


**Fig. 4.** Memory decay curve of the aversive olfactory conditioning: Changes in larval RI with time after conditioning with EA/NaCl and control conditioning with EA/DW. ***p <0.0001 with Student's *t*-test, also confirmed by Mann-Whitney *U* test, p <0.001.

In addition, *D. melanogaster* feed and lay eggs on over-ripened fruits. EA is naturally present in them and is also released in high quantities by the yeast colonies developed on these fruits. Thus, EA act as a key olfactory cue for a carbohydrate and protein-rich diet that is quite critical during the larval stage[2,4,5,13]. Thereby, associative learning with EA might be necessary for larvae for proper growth and development owing to a learned response. Although EA is present in fruits, a strong learning acquired by the larvae on its association with NaCl under laboratory conditions might signify its ecological importance for them in the wild.

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**Declarations :**

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**Conflicts of interest:** The authors have no relevant financial or non-financial interests to disclose.

**Ethics approval:** No approval of research ethics committees was required to accomplish the goals of this study because experimental work was conducted with an unregulated invertebrate species.

**Consent to participate:** Not applicable.

**Consent for publication:** Not applicable.

**Availability of data and materials:** The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

**Code availability:** Not applicable.

**Authors contribution: Md Zeeshan Ali:** Conceptualization, Writing - original draft, Methodology, Data curation, Software. **Anushree:** Software. **Jawaid Ahsan:** Validation, Supervision.

References :

1. Aceves-Pina, E. O., and W. G. Quinn, (1979). *Science*, *206*(4414): 93-96. https://doi.org/10.1126/science.206.4414.93
2. Becher, P. G., G. Flick, E. Rozpedowska, A. Schmidt, A. Hagman, S. Lebreton, M.C. Larsson, B. S. Hansson, J. Piskur, P. Witzgall and M. Bengtsson (2012). *Functional Ecology*, *26*: 822-828. https://doi.org/10.1111/j.1365-2435.2012.02006.x
3. Burke, C. J., W. Huetteroth, D. Owald, E. Perisse, M. J. Krashes, G. Das, D. Gohl, M. Silies, S. Certel, and S. Waddell, (2012). *Nature*, *492*(7429): 433-437. https://doi.org/10.1038/nature11614
4. Giang, T., J. He, S. Belaidi and H. Scholz, (2017). *Frontiers in Behavioral Neuroscience*, *11*: 160. https://doi.org/10.3389/fnbeh.2017.00160
5. Grangeteau, C., F. Yahou, C. Everaerts, S. Dupont, J. Farine, L. Beney and J. Ferveur (2018). *Scientific Reports*, *8*: 13070. https://doi.org/10.1038/s41598-018-31561-9
6. Hicklen, J., D. Jones and M. Vieyra (2010). *Journal of the South Carolina Academy of Science*, *8*(1): 9-13.
7. Honda, T., C.Y. Lee, M. Yoshida-Kasikawa, K. Honjo and K. Furukubo-Tokunaga (2014). *Scientific Reports*, *4*: 4798. https://doi.org/10.1038/srep04798

8. Honjo, K., and K. Furukubo-Tokunaga, (2009). *The Journal of Neuroscience*, *29*(3): 852-862. https://doi.org/10.1523/JNEUROSCI.1315-08.2009
9. Honjo, K., and K. Furukubo-Tokunaga, (2005). *The Journal of Neuroscience*, *25*(35): 7905-7913. https://doi.org/10.1523/JNEUROSCI.2135-05.2005
10. Khurana, S., M.B. Abu Baker and O. Siddiqi (2009). *Journal of Biosciences*, *34*(4): 621-631. https://doi.org/10.1007/s12038-009-0080-9
11. Kim, Y. C., H. G. Lee, J. Lim and K. A. Han (2013). *The Journal of Neuroscience*, *33*(4): 1672-1677. https://doi.org/10.1523/JNEUROSCI.3042-12.2013
12. Komarov, N., and S. G. Sprecher, (2022). *Fly*, *16*(1): 1-12. https://doi.org/10.1080/19336934.2021.1953364
13. Lee K. P. (2015). *Journal of Insect Physiology*, *75*, 12-19. https://doi.org/10.1016/j.jinsphys.2015.02.007
14. Lesar, A., J. Tahir, J. Wolk and M. Gershow (2021). *eLife*, *10*: e70317. https://doi.org/10.7554/eLife.70317
15. Sabandal, J. M., P. R. Sabandal, Y. Kim, and K. Han (2020). *The Journal of Neuroscience*, *40*(21): 4240-4250. https://doi.org/10.1523/JNEUROSCI.1756-19.2020
16. Thane, M., V. Viswanathan, T. C. Meyer, E. Paisios, and M. Schleyer (2019). *PloS One*, *14*(10): e0224154. https://doi.org/10.1371/journal.pone.0224154