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Acute Exposure to Arsenic Affects Cognition in *Drosophila melanogaster* Larvae

Anushree[1], Md Zeeshan Ali[1], Jawaid Ahsan[1*]

[1]Drosophila Behavioral Laboratory, Department of Biotechnology, Central University of South Bihar (CUSB), Gaya- 824 236, Bihar, India.

ABSTRACT

Arsenic has been a matter of concern and a major risk factor for public health in many parts of the world. Globally, human health and ecosystems are negatively impacted by arsenicosis of groundwater caused by natural and anthropogenic sources. Inorganic arsenic specifically in its trivalent form is highly neurotoxic due to its ability to cross the blood-brain barrier. Moreover, its exposure poses a significant threat to human health, including CNS alterations and deficits of recent memory, learning, and focus. In this study, we have explored the possibility and usefulness of the vinegar fly (commonly called fruit fly) *Drosophila melanogaster* as a model organism to investigate the effects of acute exposure to arsenic employing various behavioral assays. The vinegar fly offers the adaptability and toolkit needed for researchers to experimentally examine and study the behavior and gene expression in a defined population. We have assessed the toxic effects of inorganic arsenic on third-instar larvae of vinegar flies for their cognitive and olfactory responses in a time and dose-dependent manner. We have measured the olfactory response index of arsenic-treated flies and have also looked at their learning abilities. Our results show that acute exposure to arsenic negatively affects the olfaction, learning, and memory in the larvae of vinegar flies.

Keywords: *Drosophila melanogaster*, Arsenic, Toxicity, Behavior, Olfaction, Learning.

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Corresponding author: Jawaid Ahsan
E-mail ✉ jahsan@cub.ac.in
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INTRODUCTION

Arsenic (As) is a toxic heavy metal that can be found naturally almost everywhere [1]. Exposure to arsenic has been linked to a significantly increased risk of a wide variety of disorders, such as type II diabetes, atherosclerosis, high blood pressure, myocardial infarction, keratosis, anemia, melanoma, bladder cancer, lung cancer, and cognitive impairment [2]. Consumption of inorganic arsenic is a major contributor to an increased death rate in arsenic-endemic regions [3-5]. Arsenic poisoning is a chronic systemic disease that can develop after extended exposure to arsenic in the environment, including the air, soil, water, and foods. Arsenicosis has sparked alarm among people worldwide, and it is a problem with people's health that should not be disregarded. Arsenic poisoning poses a risk to more than 300 million people around the world, including residents of the United States, Mexico, Chile, Hungary, Bangladesh, Thailand, India, and China [6].

Various epidemiological studies have established a correlation between prolonged exposure to arsenic and cognitive impairment as well as other neurobehavioral impairments [7-11]. In rat models, arsenic, which is also a powerful neurotoxin, leads to hippocampal-dependent behavioral impairments [12]. Arsenic exposure in animal research models has been shown to produce significant changes in the functioning of the hippocampus [13]. Cognitive impairment has a negative impact on an individual's overall quality of life and can disrupt the activities that constitute a normal day. It is also related to a large number of adverse consequences, such as an increased chance of death and catastrophe [14].

The vinegar fly, *Drosophila melanogaster,* commonly known as a fruit fly has been an invaluable organism for the study of learning and memory, as well as the discovery of new genes and their functions [15]. For the past few years, a significant amount of progress has been made in unraveling the fundamental mechanisms that drive olfactory learning and memory in *Drosophila* [16, 17]. The various neurotransmitter systems like dopaminergic, GABAergic, and glutamatergic play significant roles in learning and memory [18-20]. The fruit fly is highly sensitive to odors, and the availability of robust behavioral paradigms to measure olfaction, learning, and memory makes it a useful model to study the effects of various substances on its cognitive behavior. The larvae of fruit flies are simpler in terms of neural complexity and have been used in various experiments including behavioral assays.

Making a decision is a high-level cognitive process that needs the evaluation of available options, which may be based on the decision-own maker's preferences, and prior experiences [21, 22]. Based on cognitive ability, behavioral assays are designed to determine the response of *Drosophila* larvae. The *Drosophila* larvae could also be trained and the memory formed could be studied further. In the present study, we have investigated the effects of acute exposure to arsenic in third-instar *Drosophila* larvae and measured their olfaction and learning ability in a time and dose-dependent manner using behavioral assays.

MATERIALS AND METHODS

Fly care and husbandry

Wild-type flies of the Oregon R+ strain were reared on cornmeal media and maintained at 25^0C on a 12 h light/dark cycle in a BOD incubator. The media comprised high-grade 8 gm/l agar, 15 gm/l yeast extract, 80 gm/l corn, 20 gm/l, dextrose, and 40 gm/l, sucrose. Antifungal and antibacterial agents such as 4 ml/l propionic acid and 0.6 ml/l orthophosphoric acid, respectively, were added after the media cools down to room temperature. All these chemicals were obtained from HiMedia (Mumbai, India).

Chemicals

Treatment of larvae involved the use of sodium arsenite (NaAsO2) with a purity of 90% (MW 129.91 g/mol). Different concentrations of sodium arsenite solution were made in a 5% sucrose solution used as the solvent. Monopotassium phosphate (KH_2PO_4), calcium chloride ($CaCl_2$), sodium chloride (NaCl), potassium chloride (KCl), disodium phosphate (Na_2HPO_4), and Polyethylene glycol – 6000 (PEG 6000) were used for larvae isolation. High-grade chemicals from Himedia were used for the above-mentioned experiments. The highest-quality ethyl acetate (EA) odorant and the mineral oil (diluent) were purchased from Sigma-Aldrich.

The treatment and behavioral experiments were conducted in glass Petri dishes with a diameter of 90 mm purchased from Borosil, India. Faber-Castell paint brushes with fine and soft bristles were used for handling the larvae. To isolate the larvae, strainers with fine mesh were purchased from the neighboring market.

Larvae isolation and sodium arsenite treatment

An average of 150–200 fruit flies were transferred in fresh corn meal media bottles, which were then put into the BOD incubator (25^0C) to lay eggs. After 20 hours of egg laying, the bottles were made fly-free by transferring all the flies to another bottle, and the bottles with eggs were kept at a controlled temperature of 25^0C for 3 days to continue development in the BOD incubator. The early third instar larvae were developed from eggs in around 72 h. For neurobehavioral investigations using behavioral assays, early third-instar larvae were used. These larvae were isolated by collecting the top layer of corn media gently in a strainer with the help of a soft paintbrush, taking care to prevent injury to the larvae. For the purpose of separating the larvae from the media, the coarse media containing the larvae was transferred into a vial containing 30 percent PEG-6000 solution (300 g of PEG 6000 dissolved in 1000 mL distilled water). Due to the difference in their relative densities, the media debris sank to the bottom of the vial while the larvae floated to the top. To remove the PEG solution sticking to the larval body, the top layer of the vial was emptied into the strainer and rinsed gently with running water. The larvae were then collected into a Petri plate with 0.5 mL of Ringer's solution after being removed from the cornmeal media and PEG

solution [23, 24], maintaining osmotic balance to keep larvae from drying out while behavioral assays were being performed. Ringer's solution comprised 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.

Larvae were treated with specific concentrations (1 mM and 1.5 mM) of arsenic after being harvested from media bottles as third-instar larvae. A Petri plate with a thin layer of agar was prepared by pouring 20 mL of a 1 percent Agar solution. 2.5 mL of the sodium arsenite solutions of the required concentrations were poured onto the agar layer to treat larvae. These concentrations were selected based on the study of the effect of arsenic as reported by Rizki *et al*. [25] on *Drosophila*. The harvested larvae were put onto an Agar Petri plate containing sodium arsenite solution and were treated for 17 hours. These treated larvae were then tested for olfaction, trained, and tested for memory formation.

Behavioral experiments

Larval plate assay

Odors induce varying behavioral responses in *Drosophila* via the sensitive olfactory system. The olfactory response of both untreated and arsenic-treated third instar larvae was quantified using larval plate assay. This assay was adapted from Khurana *et al*. [26]. The Petri plate contained a thin layer of 1 percent agar solution prepared in Ringer's solution. About 10 ml of the 1 percent agar solution was poured into the Petri plate. Two small round filter discs were placed diametrically opposite within the two arcs of 20 mm from the edge of the Petri plate **(Figure 1)**. On each paper disc, 20 µl of the ethyl acetate (EA) odorant diluted in mineral oil (at 10^{-2} dilution) was poured.

Approximately 50 larvae were placed at the center (S zone) of the Petri plate (90 mm) just before pouring the odor. The larvae started crawling toward the odor, after 2 minutes, the photos were taken to discern the number of larvae in various delineated zones, and the response index (RI) was calculated. The larvae were also counted manually for confirmation.

$$\text{Response Index (RI} - \text{I)} = \frac{\text{Number of larvae in zone 1 (O1)+Number of larvae in zone 2 (O2)}}{\text{Total number of larvae (O1+O2+C)}} \quad (1)$$

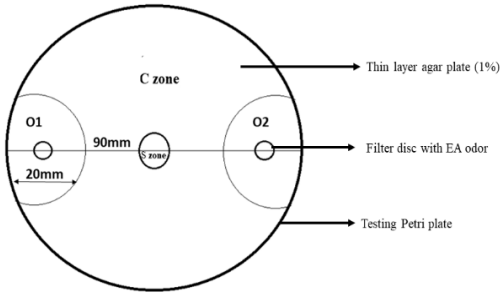


Figure 1. Schematic representation of larval plate assay to determine olfactory response index (RI). In the Start zone (S zone), approximately 50 larvae were placed. Onto the filter discs in the odor zones (O1 and O2), 20 µl of diluted EA odorant at 10-2 dilution was poured. The number of larvae in each of the designated zones was counted after 2 minutes, and RI was determined.

Larvae learning and memory assay

training

The treated and untreated larvae were trained as described by Honjo & Furukubo-Tokunaga, 2006 [27]. Untreated larvae were trained in two ways. The first set was trained in 1 ml of distilled water (DW) while the second set was trained in 1 ml of 1 M sucrose. Both DW and 1 M sucrose were spread on freshly prepared 1 percent agar Petri plates used as training plates. A filter disc was placed on the inner side of the lid and 10 µl of neat odorant (EA) was poured onto it. With the help of a paintbrush, the larvae were transferred from Ringer's solution to the training Petri plate. Immediately after the larvae were transferred, the training Petri plate was covered with the lid with the odorant. The Petri plate was left undisturbed for 30 minutes. Larvae got exposed to odor and sucrose simultaneously and associated sucrose with odor **(Figure 2)**. After training for 30 minutes, the larvae were transferred to a plate containing distilled water to rinse off any sucrose or odor residue that still adhered to their bodies. After rinsing thoroughly, the trained larvae were transferred onto the testing plate for testing their learning and memory using larval plate assay as described earlier in this paper. Arsenic-treated larvae were trained similarly as the second set of larvae mentioned above were trained with sucrose-associated odor.

Testing after training

The olfactory response of both naïve and arsenic-treated third instar larvae were quantified at 30 min time intervals for 90 min. This was the modified assay of Khurana *et al*., 2009. The response indices (RI) of trained treated and untreated larvae were calculated at 30 min of intervals for 90 min. The appetitive learning index (LI) was calculated using the following formula:

$$\mathrm{RI}-\mathrm{I} = \frac{Number\ of\ larvae\ in\ zone\ 1\ (O1) + Number\ of\ larvae\ in\ zone\ 2\ (O2)}{Total\ number\ of\ larvae\ (\mathrm{O1}+\mathrm{O2}+\mathrm{C})} \quad (2)$$

$$\text{Learning Index (LI)} = \frac{Response\ index_{sucrose} - Response\ index_{treated}}{Response\ index_{sucrose}} \quad (3)$$

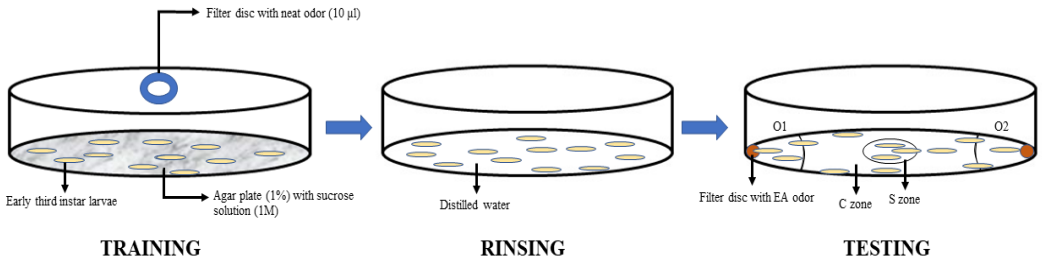


Figure 2. Schematic representation of the larval training and testing (learning and memory) assay. 90 mm Petri dish were used for training of early third instar larvae for 30 minutes. After which the larvae were rinsed with water to remove sucrose or odor stuck to their body. Finally, the trained larvae were tested in larval plate assay for 2 minutes to evaluate their response.

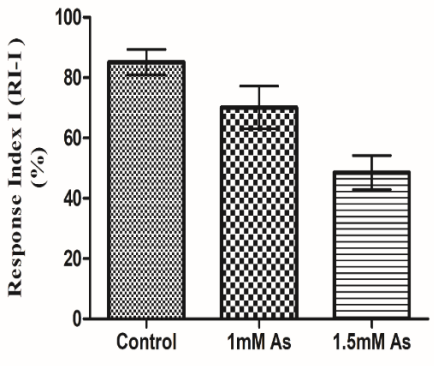
Statistics

The statistical significance of differences between RI and LI of untreated and treated larvae were estimated by parametric tests of ANOVA. Similarly, the student's *t*-test analyzed the significance of relative response relative to untreated larvae response.

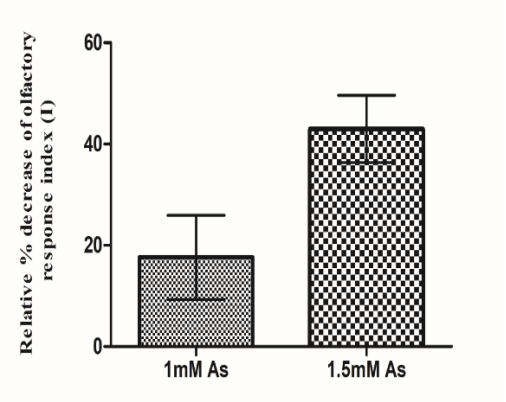
RESULTS AND DISCUSSION

Effect of arsenic on olfaction in larvae

The olfactory response in larvae to ethyl acetate (EA) odor decreased gradually with increasing concentration of arsenic treatment. The average response index (RI) of untreated larvae was measured as 85.15 percent. When larvae were exposed to arsenic at 1 mM and 1.5 mM concentrations, the average olfactory response index measured was decreased to 70.12 percent and 48.55 percent respectively. The olfactory response was not significantly affected at 1 mM arsenic treatment as compared to the untreated larvae (Control). There was a difference of 15 percent between the response of control larvae and 1 mM arsenic-treated larvae for EA. But there was a drastic change in response of 1.5 mM arsenic-treated larvae when compared to the control group. The effect of arsenic on the olfactory system of the *Drosophila* larvae was observed in their response to odor EA. The bar graph represents the olfactory response of untreated larvae and arsenic-treated larvae at 1mM and 1.5mM **(Figure 3a)**. The relative decrease in response index of treated larvae at 1mM and 1.5mM compared to control larvae were 17.63 percent and 49.93 percent respectively **(Figure 3b)**. The Mean ± SEM of 1mM arsenic-treated larvae compared to the control group was 17.63 ± 3.39. Similarly, the Mean ± SEM of 1.5mM arsenic-treated larvae compared to the control group was 42.97 ± 2.71. An average decrease of 20 percent in the olfactory response of larvae was measured when the arsenic concentration was increased from 1mM to 1.5mM.



a)



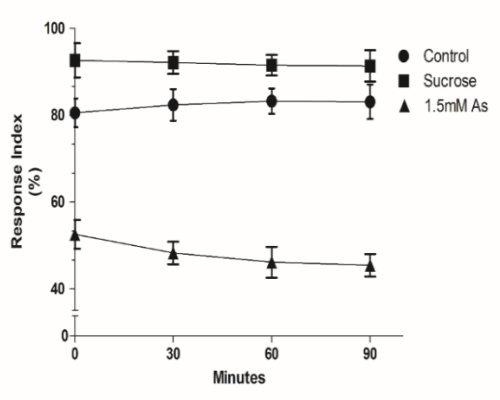
b)

Figure 3. a) The average olfactory response index I (RI-I) of untreated (Control) and arsenic (As)-treated larvae (exposed to 1mM and 1.5mM concentrations) is represented. The decrease in larvae response to odor is evident from the bar graph with the error bar representing mean ± S.D. The One-way ANOVA analysis of the statistical significance of the difference in olfactory responses of larvae resulted in ($P < 0.0001$; R square = 0.89). b) The bar graph represents the relative percentage decrease in the olfactory response (RI-I) of arsenic-treated larvae at 1mM and 1.5mM concentrations with respect to untreated larvae (Control). Student t-test analysis was performed to determine the statistical significance of the mean olfactory response with the one-tailed P value < 0.0001.

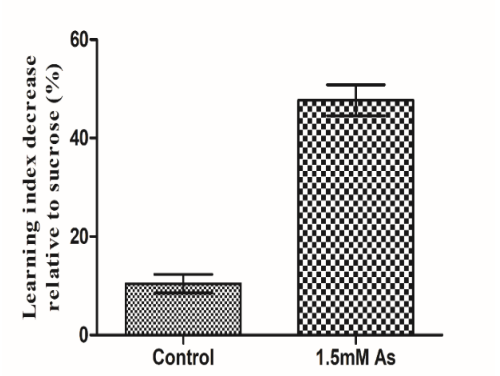
Effect of arsenic on larval learning and memory

The effect of exposure to 1.5 mM arsenic on learning and memory was studied. The average RI of Control trained (without Sucrose, only odor EA) untreated larvae towards the odor EA at 0 min (tested immediately after training) was recorded as 80.57 percent and by increasing the duration, the RI increased up to 83.11 percent at 90 minutes. The RI of Sucrose trained (Sucrose associated with odor EA) untreated larvae at 0 min was recorded as 92.64 percent and by increasing the duration, the RI decreased up to 91.37 percent. The average RI of Sucrose trained (Sucrose associated with odor EA) arsenic-treated larvae at 0 min was recorded as 52.57 percent and by increasing the duration, the RI decreased to 45.44 percent. The average response measured at each 30 min time interval is represented through the line graph for the three different larvae sets **(Figure 4a)**.

The associative learning index (LI) for untreated larvae measured at 0 min and 90 min was decreased by 13.03 percent and 9.03 percent respectively, relative to sucrose trained. Similarly, the associative learning index for treated larvae decreased by 43.24 percent and 50.26 percent at 0 min and 90 min compared to sucrose-trained larvae, which were untreated. Learning decreased to a higher extent in treated larvae compared to the control larvae. Although the sucrose-trained untreated larvae displayed maximum response to the odor, the arsenic-treated larvae' response decreased. The learning index (LI) of larvae was evaluated with respect to sucrose treated which is shown in the bar graph **(Figure 4b**).



a)



b)

Figure 4. a) Average response index of trained third instar larvae at 30 minutes intervals for ethyl acetate odor. Three different sets of larvae were used. Untreated larvae trained with water (Control), untreated larvae trained with sucrose (Sucrose), and arsenic-treated trained with sucrose (1.5mM As). One-way ANOVA statistically determined the significant difference between the three different sets of larvae responses with P value < 0.0001 and R square = 0.99. b) Learning index decrease in the percentage of untreated (Control) and arsenic-treated (1.5mM As) flies relative to sucrose flies. The error bar represents the Mean ± S.D for each column. One-tailed student's t-test was used to determine the significant difference between the control and 1.5 mM As group of third instar larvae learning index with ***P < 0.0001 and 95 percent confidence interval.

Trace elements, like Arsenic, found in nature play crucial roles in the metabolic processes of all living things [28, 29]. Arsenic in its inorganic form is highly toxic. As a consequence, changes in inorganic arsenic levels can cause adverse effects on the brain, including cognitive behavior impairment, neurological disorder acceleration, and dysfunction of hippocampal regions [13]. Based on skin cancer risk factors due to arsenic, the Food and Agriculture Organization specified arsenic concentration for safe drinking water as 10 µg/l. Although the actual value for arsenic in drinking water estimated by WHO is 0.17 µg/l [30]. According to reports, arsenic exposure levels have gone above the allowable limits [31-33].

In this study, we found that arsenic exposure is associated with cognitive impairment, and arsenicosis could influence cognitive performance independently. The olfactory response of early third-instar larvae was significantly affected upon getting exposed to arsenic. Also, as the concentration of arsenic was increased, the olfactory response index (RI) decreased further. The findings of this study clearly indicate that the neural circuit for sensing the odor gets affected due to arsenic exposure. Consequently, the response of arsenic-treated larvae toward ethyl acetate odor decreased as compared to untreated larvae.

According to studies conducted on mice, arsenic reduces the N-methyl-D-aspartate receptor (NMDA) receptors in the hippocampus region of the brain, which is crucial for synaptic plasticity, learning, and memory [34, 35]. The present study also correlated similar trends in the ability of larvae for associative learning revealing the neurotoxic effect of arsenic. In this study, we found that the response of arsenic-treated larvae decreased with increasing duration after training. Learning associated with sucrose was maximum in untreated larvae but in treated larvae, it decreased remarkably in a time-dependent manner. Kumar *et al*. [36] reported that children drinking arsenic-contaminated water displayed hyperactivity, loss of concentration, and alertness. They also hypothesized that arsenic acts as a xenoestrogen, which affects endocrine function and generates free radicals that decrease dopamine secretion, which in turn results in deteriorated brain growth and behavioral impairment. Another study by Wang *et al*. [37] in China concluded that arsenic exposure-related cognitive impairment in a young population is a sign of eventual dementia or more severe cognitive disorders. Our results show that acute exposure to arsenic affects the neural circuits involving sensing odor, learning, and memory. Thus, fruit flies could be used as a useful model to study such effects at different levels up to the molecular levels to know the genes and proteins being affected due to exposure to arsenic.

CONCLUSION

Our results show that the olfactory and learning abilities in third-instar *Drosophila* larvae are dramatically decreased by acute exposure to arsenic in a time- and dose-dependent manner. This is the first report on the toxic effects of arsenic on fruit fly larvae's cognitive abilities. To fully comprehend the molecular causes of arsenic-induced reduced cognitive abilities in larvae, more investigations are needed.

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CONFLICT OF INTEREST: None

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ETHICS STATEMENT: None

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