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Cognitive Impairments Induced by Acute Arsenic Exposure in *Drosophila melanogaster* Larvae

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ABSTRACT

Arsenic has become a significant concern due to its detrimental effects on public health across various regions globally. Both natural and human-made sources contribute to groundwater contamination, leading to arsenicosis, which adversely affects ecosystems and human health. The trivalent form of inorganic arsenic is particularly harmful because it is neurotoxic and capable of crossing the blood-brain barrier. This exposure poses substantial health risks, particularly to the central nervous system (CNS), including impairments in recent memory, learning, and attention. In this study, we investigate the potential of using the *Drosophila melanogaster*, commonly known as the vinegar fly, as a model organism to study the effects of short-term arsenic exposure through various behavioral tests. The vinegar fly is a versatile organism that provides the necessary tools for researchers to investigate behavioral changes and gene expression in controlled populations. We focused on the toxic effects of inorganic arsenic on third-instar larvae and assessed their cognitive and olfactory responses in a time- and dose-dependent manner. By measuring the olfactory response index and assessing learning capabilities, we found that acute arsenic exposure significantly disrupts olfaction, learning, and memory in the larvae of vinegar flies.

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

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Introduction

Arsenic (As) is a harmful heavy metal found ubiquitously in the environment [1]. Its exposure has been associated with an elevated risk of numerous health issues, including type II diabetes, atherosclerosis, hypertension, heart attacks, melanoma, keratosis, anemia, lung cancer, bladder cancer, and cognitive decline [2]. The ingestion of inorganic arsenic contributes significantly to higher mortality rates in regions where arsenic contamination is prevalent [3-5]. Chronic exposure to arsenic from various sources such as air, water, soil, and food can lead to arsenicosis, a systemic condition. This has raised significant health concerns worldwide, affecting over 300 million people across countries including the United States, Hungary, Chile, Mexico, India, Bangladesh, Thailand, and China [6].

Numerous epidemiological studies have highlighted a link between long-term arsenic exposure and neurocognitive deficits, alongside other neurobehavioral impairments [7-11]. In rodent models, arsenic has been displayed to disrupt hippocampal functions, leading to behavioral impairments dependent on hippocampal activity [12]. Research on arsenic exposure in animal models has revealed considerable alterations in hippocampal function [13]. Cognitive deficits not only degrade an individual's quality of life but also increase the likelihood of premature death and other severe health consequences [14].

Drosophila melanogaster, the vinegar fly or fruit fly, has long been an essential model organism for studying memory and learning as well as for uncovering new genes and their roles [15]. Significant strides have been made in understanding the basic mechanisms underlying olfactory learning and memory in *Drosophila* over recent years [16, 17]. Various neurotransmitter systems, including GABAergic, dopaminergic, and glutamatergic pathways, are crucial for these cognitive processes [18-20]. The vinegar fly is highly sensitive to odors, and the availability of effective behavioral assays to measure olfaction, learning, and memory makes it an ideal model for investigating the impact of different substances on cognitive function. Furthermore, the simpler neural structure of *Drosophila* larvae makes them an excellent subject for behavioral experiments.

Decision-making, a complex cognitive process, involves evaluating options based on an individual's preferences and past experiences [21, 22]. Behavioral assays are employed to assess the responses of *Drosophila* larvae based on their cognitive abilities. These larvae can be trained, and the formation of memory can be analyzed. In this study, we explore the effects of acute arsenic exposure on third-instar *Drosophila* larvae, examining their olfactory and learning abilities in a dose- and time-dependent manner using behavioral assays.

Materials and Methods

Fly care and maintenance

Wild-type Oregon R+ strain fruit flies were maintained on a cornmeal-based medium and kept in a controlled environment at 25 °C with a 12-hour light/dark cycle in a BOD incubator. The medium consisted of high-quality ingredients: 8 g/l agar, 15 g/l yeast extract, 80 g/l corn, 20 g/l dextrose, and 40 g/l sucrose. After cooling to room temperature, antifungal and antibacterial agents, such as 4 ml/l propionic acid and 0.6 ml/l orthophosphoric acid, were added. All chemicals were sourced from HiMedia (Mumbai, India).

Chemicals

Sodium arsenite (NaAsO2) with 90% purity (MW 129.91 g/mol) was used for larvae treatment. Solutions of sodium arsenite were prepared in a 5% sucrose solution. For larvae isolation, monopotassium phosphate (KH2PO4), sodium chloride (NaCl), calcium chloride (CaCl2), disodium phosphate (Na2HPO4), potassium chloride (KCl), and polyethylene glycol 6000 (PEG 6000) were used, all sourced from HiMedia. Ethyl acetate (EA) odorant and mineral oil (diluent) were purchased from Sigma-Aldrich.

The behavioral assays were performed in 90 mm diameter glass Petri dishes (Borosil, India), and larvae were handled using soft-bristled Faber-Castell paint brushes. Fine-mesh strainers for larvae isolation were acquired locally.

Larvae isolation and sodium arsenite treatment

Approximately 150–200 adult fruit flies were placed in fresh cornmeal media bottles and incubated at 25 °C for egg laying. After 20 hours, the adult flies were removed, and the egg-laden bottles were left in the BOD incubator at 25 °C for 3 days for larvae development. Early third-instar larvae, emerging within 72 hours, were collected for the behavioral assays. To isolate the larvae, the top layer of the cornmeal media was gently collected with a fine mesh strainer and soft paintbrush to avoid injury. The larvae-containing media was transferred to a vial containing a 30% PEG-6000 solution (300 g of PEG 6000 in 1000 mL distilled water). Due to differences in density, the larvae floated to the top, while the media debris sank. After rinsing the larvae with running water to remove the PEG solution, they were collected in a Petri dish with 0.5 mL of Ringer’s solution, which contained 128 mM NaCl, 4.7 mM KCl, 1.8 mM CaCl2, 0.9 mM Na2HPO4, and 0.37 mM KH2PO4 to maintain osmotic balance and prevent desiccation during the behavioral testing.

For treatment, third-instar larvae were exposed to 1 mM and 1.5 mM concentrations of sodium arsenite. A Petri dish with a thin layer of 1% agar (20 mL) was prepared, and 2.5 mL of the sodium arsenite solutions were applied to the agar surface. These concentrations were based on previous studies on the effects of arsenic on *Drosophila* [23]. The treated larvae were incubated on the agar Petri dish for 17 hours before undergoing olfactory testing, memory training, and cognitive assessment.

Behavioral experiments

Larval plate assay

The olfactory system of *Drosophila* triggers different behavioral responses when exposed to various odors. To measure the olfactory response in both untreated and arsenic-exposed third-instar larvae, the larval plate assay was used, following a modified method from Khurana *et al*. [24]. In this assay, a thin layer of 1% agar solution in Ringer's solution was prepared and poured into a Petri dish. A total of 10 milliliters of the solution was added to the Petri dish. Two small circular filter paper discs were placed opposite each other, positioned within 20 mm arcs from the edge of the Petri plate **(Figure 1)**. Each disc was treated with 20 µl of ethyl acetate (EA) odorant, which was diluted in mineral oil at a 10^-2 ratio.

Around 50 larvae were placed in the center (S zone) of the Petri dish (90 mm diameter) just before the odor was introduced. After two minutes, the larvae began moving toward the odor. Photographs were taken to observe and count the number of larvae in different predefined zones, and the response index (RI) was calculated. The larvae were also counted by the researcher to verify the results.

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

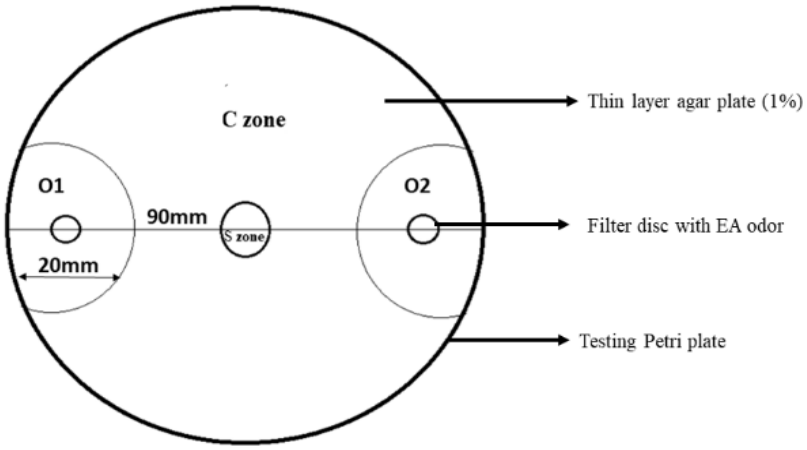


Figure 1. A schematic diagram illustrating the larval plate assay for assessing the olfactory response index (RI); around 50 larvae were initially placed in the designated start zone (S zone); to create an odor gradient, 20 µl of ethyl acetate (EA) odorant, diluted to 10^-2, was applied to filter paper discs positioned in the odor zones (O1 and O2); after a 2-minute interval, the number of larvae in each marked zone was recorded, and the RI was determined.

Larvae learning and memory assay

Training

For the training phase, both arsenic-treated and untreated larvae followed a method adapted from Honjo and Furukubo-Tokunaga [25]. The untreated larvae were trained in 2 separate conditions. In the first condition, they were trained using 1 milliliter of distilled water (DW), and in the second condition, they were trained with 1 ml of 1 M sucrose. Both solutions were spread onto freshly prepared 1% agar plates, which were used as training surfaces. A filter paper disc was placed inside the lid of the Petri dish, and 10 µl of undiluted ethyl acetate (EA) odorant was applied to the disc. Using a paintbrush, the larvae were carefully transferred from Ringer's solution to the training plate. Once the larvae were on the plate, it was sealed with the lid containing the odorant. The setup remained undisturbed for half an hour, during which the larvae simultaneously encountered the odor and sucrose, forming an association between the two **(Figure 2)**. After training, the larvae were transferred to a plate with distilled water to wash off any remaining sucrose or odorant. Once rinsed, the larvae were placed on a testing plate for further behavioral assessment. For the arsenic-treated larvae, the training was conducted in the same way, using sucrose-associated odor, just as in the second group of untreated larvae.

Post-training testing

The olfactory responses of both untreated and arsenic-exposed third-instar larvae were evaluated at 30-minute intervals over 90 minutes, following a modification of the protocol by Khurana *et al*. [24]. The response indices

(RI) for the trained larvae, both treated and untreated, were calculated at these 30-minute intervals. Additionally, the appetitive learning index (LI) was determined using the following formula:

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

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

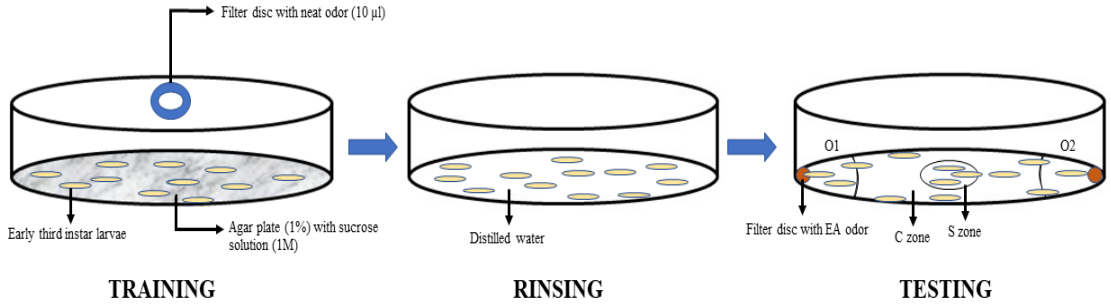


Figure 2. Schematic representation of the larval training and testing assay for evaluating learning and memory; early third-instar larvae underwent a 30-minute training session in a 90 mm Petri dish; following training, the larvae were rinsed with water to eliminate any residual sucrose or odor molecules attached to their bodies; subsequently, the trained larvae were subjected to a 2-minute larval plate assay to measure their olfactory response.

Statistics

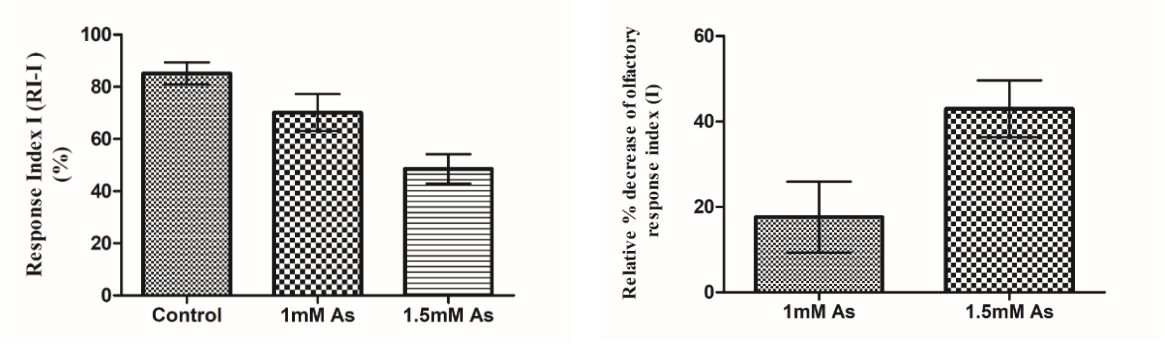
To assess the significance of variations in response indices (RI) and learning indices (LI) between untreated and arsenic-exposed larvae, parametric ANOVA tests were employed. Additionally, a Student's t-test was conducted to determine the significance of the relative response of treated larvae compared to their untreated counterparts.

Results and Discussion

Impact of arsenic on larval olfactory response

Exposure to arsenic led to a progressive reduction in the olfactory sensitivity of larvae toward ethyl acetate (EA) odor. The RI for the control group (untreated larvae) was recorded at 85.15%. Upon exposure to arsenic at concentrations of 1 mM and 1.5 mM, the RI values declined to 70.12% and 48.55%, respectively. While the response of larvae subjected to 1 mM arsenic treatment did not show a statistically significant deviation from the control group, a notable difference of 15% was noticed in their ability to detect EA. In contrast, larvae treated with 1.5 mM arsenic exhibited a substantial decline in olfactory response compared to the control.

The influence of arsenic exposure on the olfactory system of *Drosophila* larvae was reflected in their reaction to EA odor. A bar graph **(Figure 3a)** illustrates the RI values of untreated and arsenic-treated larvae at both concentrations. The relative reduction in RI for larvae exposed to 1 mM and 1.5 mM arsenic was found to be 17.63% and 49.93%, respectively **(Figure 3b)**. The Mean ± SEM for 1 mM arsenic-treated larvae in comparison to the control was 17.63 ± 3.39, whereas for 1.5 mM arsenic-treated larvae, it was 42.97 ± 2.71. Overall, an approximate 20% decline in the olfactory response was observed as the arsenic concentration increased from 1 mM to 1.5 mM.



a) b)

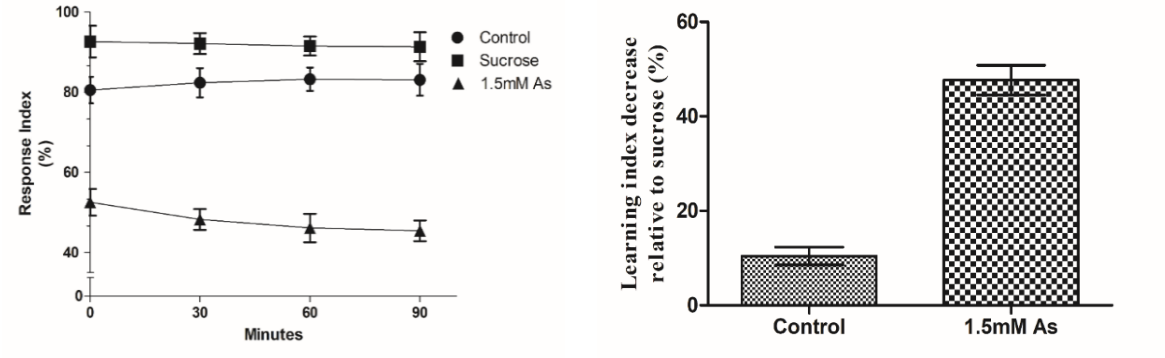
Figure 3. a) bar graph displaying the average olfactory response index I (RI-I) for both untreated (control) and arsenic (As)-treated larvae exposed to 1 mM and 1.5 mM concentrations; the decline in olfactory response due to arsenic exposure is depicted, with error bars indicating mean ± S.D; one-way ANOVA statistical analysis demonstrated a significant difference in olfactory responses among groups ($P < 0.0001$; $R^2 = 0.89$); b) a bar graph illustrating the percentage reduction in olfactory response (RI-I) in arsenic-treated larvae (1 mM and 1.5 mM) relative to the untreated control group; statistical significance in mean olfactory response was evaluated using a one-tailed student's t-test, yielding a P-value of < 0.0001.

Effect of arsenic on larval learning and memory

The impact of 1.5 mM arsenic exposure on memory and learning was assessed in third-instar larvae. The average response index (RI) of the Control trained group (exposed only to ethyl acetate (EA) odor without sucrose) was recorded at 80.57% immediately after training (0 min). Over time, the RI showed a slight increase, reaching 83.11% in one hour and a half. In contrast, the RI for the sucrose-trained group (sucrose paired with EA odor) started at 92.64% at 0 min and showed a marginal decline to 91.37% over the 90 minutes.

For arsenic-exposed larvae trained with sucrose-associated odor, the RI was considerably lower, starting at 52.57% at 0 min and further declining to 45.44% at 90 minutes. A line graph illustrating the response trends for all three groups at 30-minute intervals is provided **(Figure 4a)**.

The associative learning index (LI) of untreated larvae, when measured at 0 min and 90 min, showed a reduction of 13.03% and 9.03%, respectively, in comparison to the sucrose-trained group. In arsenic-treated larvae, the LI decline was more pronounced, with reductions of 43.24% at 0 min and 50.26% at 90 min compared to untreated sucrose-trained larvae. This data suggests that arsenic exposure significantly impairs associative learning, with a greater reduction in learning ability observed in treated larvae relative to controls. While untreated sucrose-trained larvae demonstrated the strongest response to the odor, arsenic-exposed larvae exhibited a marked decline in response. The comparative learning index (LI) values, assessed concerning sucrose training, are visualized in the bar graph **(Figure 4b)**.



a) b)

Figure 4. a) the average response index (RI) of third-instar larvae trained for ethyl acetate (EA) odor was measured at 30-minute intervals; three experimental groups were analyzed: untreated larvae trained in water (control), untreated larvae trained with sucrose (sucrose), and arsenic-exposed larvae trained with sucrose (1.5mM As); statistical analysis using one-way ANOVA confirmed a significant difference in response

among the groups, with a P-value < 0.0001 and an R square value of 0.99; b) the percentage reduction in the learning index (LI) of untreated (control) and arsenic-treated (1.5 mM As) larvae was compared to the sucrose-trained group. The error bars indicate the Mean ± S.D. statistical significance between the control and 1.5 mM as-treated larvae was determined using a one-tailed student's t-test, with ***P < 0.0001 and a 95% confidence interval.

Effect of arsenic on cognitive function and olfactory response

Trace elements such as arsenic are naturally occurring and play essential roles in biological processes across all living organisms [26, 27]. However, its inorganic form is very toxic, and fluctuations in inorganic arsenic levels can lead to severe neurological consequences. These include cognitive impairments, accelerated progression of neurological disorders, and disruptions in hippocampal function [13]. Due to the increased risk of skin cancer associated with arsenic, the Food and Agriculture Organization has set the safe drinking water threshold at 10 µg/l, although the World Health Organization (WHO) has estimated an even lower value of 0.17 µg/l [28]. Reports suggest that arsenic contamination in drinking water has exceeded these recommended limits in various regions [29-31].

The results of this study indicate a strong correlation between arsenic exposure and cognitive decline, demonstrating that arsenicosis can independently impact cognitive performance. A significant reduction in the olfactory response of early third-instar larvae was observed upon arsenic exposure, with the response index (RI) progressively decreasing as arsenic concentration increased. These results suggest that arsenic exposure disrupts the neural circuitry responsible for odor detection, thereby impairing the ability of larvae to respond to ethyl acetate odor compared to the untreated group.

Prior research on mice has shown that arsenic exposure reduces N-methyl-D-aspartate (NMDA) receptor expression in the hippocampus—critical for learning, synaptic plasticity, and memory formation [32, 33]. Our study aligns with these findings, as arsenic-treated larvae exhibited a decline in associative learning, reinforcing arsenic's neurotoxic effects. Additionally, a time-dependent reduction in learning ability was observed, with untreated larvae displaying maximum sucrose-associated learning, whereas arsenic-exposed larvae showed a significant decline over time.

Kumar *et al*. [34] mentioned that children exposed to arsenic-contaminated drinking water displayed behavioral deficits, including hyperactivity, reduced concentration, and diminished alertness. They proposed that arsenic acts as a xenoestrogen, disrupting endocrine function and generating free radicals that reduce dopamine secretion, ultimately impairing brain development and behavior. Similarly, a study conducted by Wang *et al*. [35] in China linked arsenic exposure in young individuals to cognitive impairments, suggesting a potential progression toward dementia or other severe neurological disorders.

The results of this study reinforce that acute arsenic exposure negatively affects neural circuits associated with odor detection, learning, and memory. Given these results, *Drosophila melanogaster* can serve as a valuable model for investigating arsenic-induced neurotoxicity at multiple levels, including molecular mechanisms that influence gene and protein expression in response to arsenic exposure.

Conclusion

This study demonstrates that acute arsenic exposure significantly impairs both olfactory function and learning capacity in third-instar Drosophila larvae, with the extent of these effects increasing in a time- and dose-dependent manner. To our knowledge, this study is the only one to report the detrimental impact of arsenic toxicity on the cognitive abilities of fruit fly larvae. Further research is necessary to elucidate the molecular mechanisms underlying arsenic-induced cognitive decline in larvae.

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Conflict of Interest: None

Financial Support: None

Ethics Statement: None

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