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Chronic Exposure to Lead Acetate Affect Cognition in *Drosophila melanogaster* Larvae

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Abstract

Lead toxicity has been a matter of concern and a major risk factor for public health in many parts of the world. Lead (Pb) is a toxic heavy metal that is widely dispersed in the environment due to various human activities such as industrial emissions and the use of lead-based products. Inorganic Lead 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 chronic exposure to lead acetate 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 lead acetate 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 lead-treated flies and have also looked at their learning abilities. Our results show that chronic exposure to lead acetate negatively affects the olfaction in the larvae of vinegar flies. Most behavioural parameters investigated were significantly rescued in the lead-poisoned fruit flies ($p < 0.05$) when co-treated with antioxidant Butylated hydroxyanisole. The results suggest that Butylated hydroxyanisole has a protective effect against lead toxicity in *Drosophila melanogaster* and could potentially be used as a therapeutic agent for lead poisoning in humans.

Keywords: Lead Toxicity, Drosophila melanogaster, Cognition, Olfaction, Butylated Hydroxyanisole (BHA).

Introduction

Thousands of chemicals are in commercial use today, with more being introduced each year for which there is little or no toxicological data. Furthermore, we continue to be exposed to the increasing environmental stress caused by persistent toxicants without any knowledge of its harmful effects (Rand, 2010) Heavy metals form a major causative group with cognitive and neurological implications of exposure. Excess accumulation of few heavy metals like Copper and Zinc which are otherwise of nutritional and physiological importance; and non-essential toxic metals like Lead and Mercury is detrimental (Hapke *et al.* 1987; Yepiscoposyan *et al.* 2006). Studies have shown that the effects of chronic developmental exposure of these toxins are more pronounced than the acute adult exposure (Hirsch *et al.,* 2003). This may be due to its direct effects on the developing neuronal and endocrine systems that regulate fitness and behaviour in adults. Even sub-lethal doses of toxins are capable of altering the physiological mechanisms qualitatively and quantitatively as shown by (Hirsch *et al* 2003) in fruit flies.

The presence of heavy metals poises significant environmental pollution and acts as a causative agent to myriads of diseases including kidney dysfunction (Tsai *et al.,* 2017) [19], nervous system disorder, cardiovascular diseases (Obeng-Gyasi *et al.,* 2018) [14], gastrointestinal diseases (Balali Mood *et al.,* 2021) [2]. Unlike organic matter, heavy metals do not decompose within a short time but rather accumulate in organs such as the kidney, liver, brain, and bone, which eventually leads to chronic poisoning and death (Rehman *et al*., 2018; Cheng *et al*., 2019) [17, 4]. Lead (Pb) is a toxic heavy metal that is widely dispersed in the environment due to various human activities such as industrial emissions and the use of lead-based products. Lead toxicity is known to cause a wide range of negative effects on human health, including reproductive toxicity, neurological damage, and cardiotoxicity, with oxidative stress being the likely mechanism of action of this environmentally persistent toxicant (Flora *et al*., 2012; Rehman *et al*., 2018; Cheng *et al*., 2019) [6, 17, 4]. Lead in the environment enters human or animal bodies mostly via ingestion or inhalation.

Lead toxicity occurs both in children and adults, with a worldwide burden of childhood lead exposure estimated to cost 998 billion US dollars annually or 1.47% of the world's GDP in 2011 with lower- and middle-income countries

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(LMICs) bearing the brunt of the costs (IHME, 2017; Burki, 2020) [10, 3]. The Institute of Health Measurement and Evaluation (IHME) estimates that due to the long-term adverse effects of lead exposure, 1.06 million people died, and 24.4 million people suffered morbidity in 2017, and lead induced idiopathic developmental intellectual disability accounted for 63.2% of the global burden in 2016, with low and middle-income countries bearing the heaviest burden (Li *et al*., 2020). The presence of lead in gasoline, jewelry, traditional medications, toys, and foods have led to an increased worldwide environmental burden (Obeng Gyasi, 2018) [15]. Given the growing concern over the negative health impacts of lead toxicity, it is crucial to identify potential strategies for ameliorating its toxic effects.

Butylated hydroxyanisole (BHA) is a mixture of isomers of tertiary butyl-substituted 4-methoxphenols. BHA is a synthetic antioxidant. Cosmetic grade BHA consists chiefly of 3-t-butyl-4-hydroxyanisole (3-BHA) with lesser amounts of 2-t-butyl- 4-hydroxyanisole (2-BHA). (Estrin, 1977). BHA exhibits antioxidant properties and acts synergistically with acids, butylated hydroxytoluene (BHT), propyl gallate, hydroquinone, methionine, lecithin, and thiodipropionic acid in protecting lipids against autoxidation. (Windholz, 1976 and Surak, 1974) [20, 18].

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 (Keene and Waddell, 2007) [11]. 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* (McGuire *et al*., 2005; Gervasi *et al*., 2010) [13]. The various neurotransmitter systems like dopaminergic, GABAergic, and glutamatergic play significant roles in learning and memory (Aso *et al*., 2010; Zhou *et al*., 2019) [1, 4].

The fruit fly highly sensitive to odors, and the availability of robust behavioral paradigms to measure olfaction 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 (Gold *et al*., 2007; Shadlen *et al*., 2013) [7]. Based on cognitive ability, behavioral assays are designed to determine the response of *Drosophila* larvae. In the present study, we have investigated the effects of chronic exposure to lead in third-instar *Drosophila* larvae and measured their olfaction ability in a time and dose-dependent manner using behavioral assays.

Materials and Methods

Establishment of Stock

Wild type *Drosophila melanogaster*- Oregon K strain (OK) was obtained from *Drosophila* stock centre, University of Mysore. Flies were grown and aged in culture bottles/vials on wheat cream agar media (100 Soji, 100g jaggery, 10g agar and 7.5ml propionic acid in 1dm3 distilled water) with regular subculturing and maintained for all experiments at 24 ^{0}C with 60-70% relative humidity and ambient lighting condition with a sprinkle of live Baker's yeast. All collection of virgin, adult flies were performed under brief anesthesia. Dose administration was achieved via larval feeding for all treatments.

Heavy Metal Treatment

For the convenient establishment of treatment group.. Eggs collected from the OK cultures by modified method of Delcour (Ramachandra and Ranganath, 1988) [16] were distributed on the culture medias with 1.0mM, 1.5mM Lead acetate (SDFCL, Mumbai) and Antioxidant (BHA) 0.9 mM in a media. 5 replicates per concentration were maintained for both compounds.

The highest-quality ethyl acetate (EA) odorant and the mineral oil (diluent). The treatment and behavioral experiments were conducted in glass Petri dishes with a diameter of 90 mm. 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.

Behavioral Experiments (Anushree *et al.*)

Larval Plate Assay

Response Index (RI − I)= $\frac{\text{Number of larvae in zone 1 (O1)+Number of larvae in zone 2 (O2)}}{\text{Total number of larvae (O1+O2+C)}}$ x100

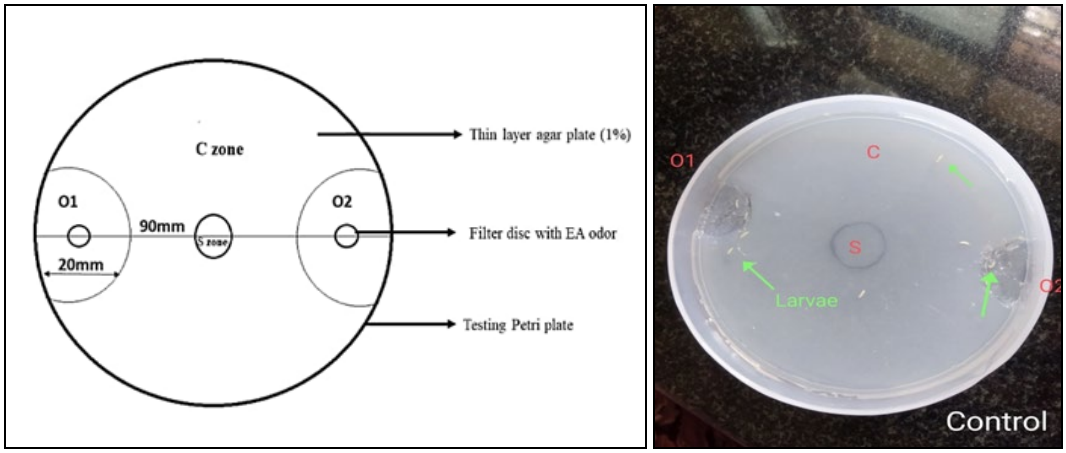

Fig 1: Schematic representation of larval plate assay to determine olfactory response index (RI).

In the Start zone (S zone), approximately 10 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.

The treated and untreated larvae were trained as described by (Honjo & Furukubo-Tokunaga, 2009) [9]. 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 Odors induce varying behavioral responses in *Drosophila* via the sensitive olfactory system. The olfactory response of both untreated and Lead acetate treated third instar larvae was quantified using larval plate assay. This assay was adapted from (Khurana *et al*. 2009) [12]. 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 10 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.

Statistical Analysis

The data obtained were analyzed using IBM SPSS version 29.0. Mean, standard error, one way ANOVA, and Tukey's Post-Hoc test were carried out for the data obtained for olfactory assay. Significance of relative response relative to untreated larvae response.

Results and Discussion

Effect of Lead Acetate on Olfaction in Larvae

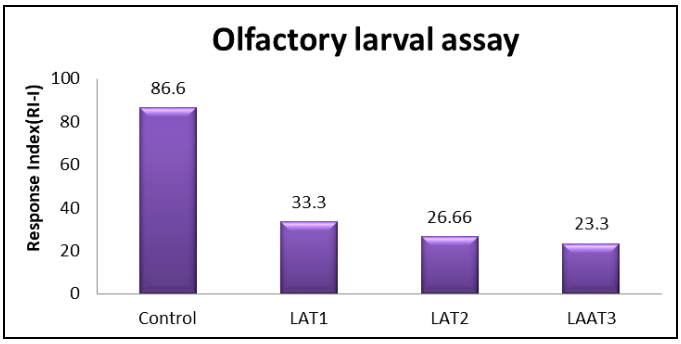

Fig 2: The average olfactory response index I (RI-I) of untreated (Control) and lead acetate-treated larvae (exposed to 1mM and 1.5mM and antioxidant concentrations) is represented.

Fig.3 reveals the effect of lead acetate on the olfactory assay of *D.melanogaster* raised in control diet and treated media. According to the data obtained the larval plate assay was found to be high in lead acetate treated media compared to control. In between the concentration groups of control media which is significant with $p < 0.05$, $df = 2$ and $F = 18.60$. Fig.4 reveals the effect of lead acetate on the olfactory assay of *D.melanogaster* raised in control diet and treated media. According to the data obtained the larval plate assay was found to be high in lead acetate treated media compared to control. In between the concentration groups of LAT1 media which is significant with $p < 0.05$, $df = 2$ and $F = 75.0$. Fig.5 reveals the effect of lead acetate on the olfactory assay of *D.melanogaster* raised in control diet and treated media. According to the data obtained the larval plate assay was found to be high in lead acetate treated media compared to control. In between the concentration groups of LAT2 media which is significant with $p < 0.05$, $df = 2$ and $F = 108.0$. Fig.6 reveals the effect of lead acetate on the olfactory assay of *D.melanogaster* raised in control diet and treated media. According to the data obtained the larval plate assay was found to be high in antioxidant treated media compared to control. In between the concentration groups of LAAT3 media which is significant with $p < 0.05$, $df = 2$ and $F = 64.5$.

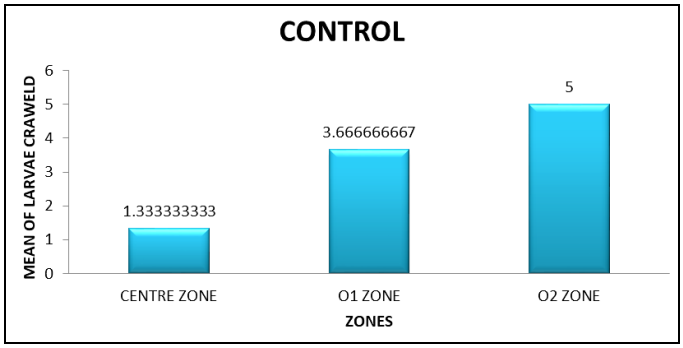

Fig 3: Number of larvae crawled in control groups of *D.melanogaster.*

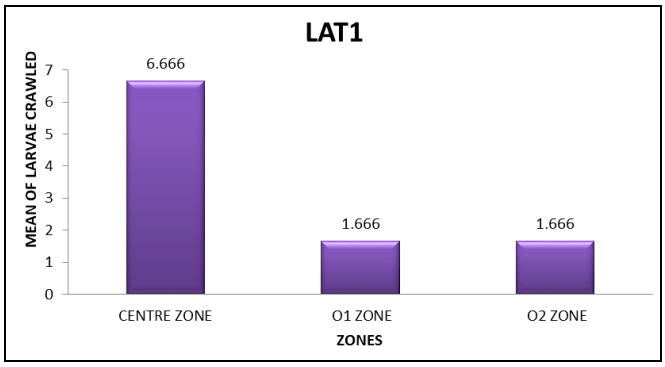

Fig 4: Number of larvae crawled in LAT1 groups of *D.melanogaster.*

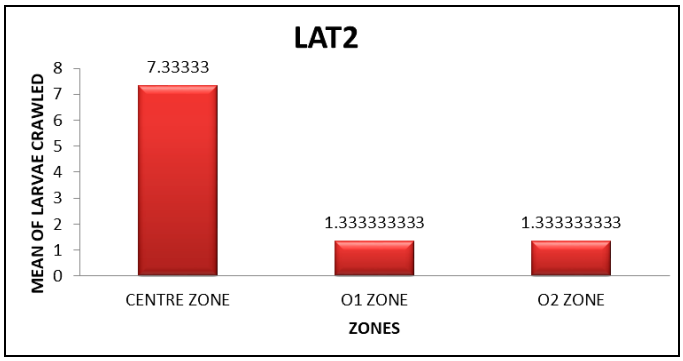

Fig 5: Number of larvae crawled in LAT2 groups of *D.melanogaster*

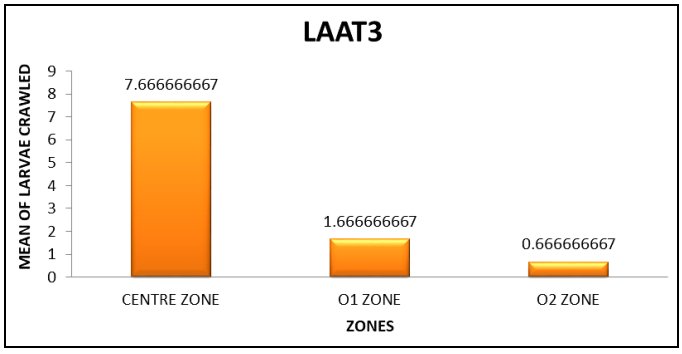

Fig 6: Number of larvae crawled in LAAT3 groups of *D.melanogaster.*

Discussion

The study investigates the impact of lead acetate and dried BHA on the olfactory assay of *Drosophila melanogaster* (fruit flies). The effects were compared across control diets and various concentrations of lead acetate media.

Fig.3 reveals the effect of lead acetate on the olfactory assay of *D.melanogaster* raised in control diet and treated media. According to the data obtained the larval plate assay was found to be high in lead acetate treated media compared to control. In between the concentration groups of control media which is significant with $p < 0.05$, $df = 2$ and $F = 18.60$. Fig.4 reveals the effect of lead acetate on the olfactory assay of *D.melanogaster* raised in control diet and treated media. According to the data obtained the larval plate assay was found to be high in lead acetate treated media compared to control. In between the concentration groups of LAT1 media which is significant with $p < 0.05$, $df = 2$ and $F = 75.0$. Fig.5 reveals the effect of lead acetate on the olfactory assay of *D.melanogaster* raised in control diet and treated media. According to the data obtained the larval plate assay was found to be high in lead acetate treated media compared to control. In between the concentration groups of LAT2 media which is significant with $p < 0.05$, $df = 2$ and $F = 108.0$. Fig.6 reveals the effect of lead acetate on the olfactory assay of *D.melanogaster* raised in control diet and treated media. According to the data obtained the larval plate assay was found to be high in antioxidant treated media compared to control. In between the concentration groups of LAAT3 media which is significant with $p < 0.05$, $df = 2$ and $F = 64.5$. The olfactory response in larvae to ethyl acetate (EA) odor decreased gradually with increasing concentration of lead acetate treatment. The average response index (RI) of untreated larvae was measured as 86.6 percent. When larvae were exposed to lead acetate at 1 mM and 1.5 mM concentrations, the average olfactory response index measured was decreased to 33.3 percent and 26.6 percent respectively. When larvae were exposed to antioxidant BHA, the average olfactory response index measured was decreased to 23.3 percent. The olfactory response was not significantly affected at 1 mM lead acetate treatment as compared to the untreated larvae (Control). There was a difference of 53.3 percent between the response of control larvae and 1 mM lead-treated larvae for EA. But there was a drastic change in response of 1.5 mM lead-treated larvae when compared to the control group. The effect of lead on the olfactory system of the *Drosophila* larvae was observed in their response to odor EA. The Mean ± SEM of 1mM lead acetate treated larvae compared to the control group was 25.0 ± 0.84. Similarly, the Mean ± SEM of 1.5mM lead acetate treated larvae compared to the control group was 36.0 ± 1.01. The Mean ± SEM of lead acetate and antioxidant treated larvae compared to the control group was 43.0 ± 1.11. (figure.2)

In this study, we found that lead exposure is associated with cognitive impairment could influence cognitive performance independently. The olfactory response of early third-instar larvae was significantly affected upon getting exposed to lead. Also, as the concentration of lead acetate 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 lead exposure. Consequently, the response of lead acetate-treated larvae toward ethyl acetate odor decreased as compared to untreated larvae.

The present study also correlated similar trends in the ability of larvae for associative learning revealing the neurotoxic effect of lead acetate. Reported that children drinking lead contaminated water displayed concentration, and hyperactivity, alertness. Loss of They also hypothesized that lead 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.

Conclusion

The present study demonstrates that exposure to lead acetate at concentrations of 1.0 mM and 1.5 mM significantly impairs olfactory responses in larvae, as evidenced by reduced attraction to odorant cues in behavioral assays. The higher concentration (1.5 mM) elicited a more pronounced olfactory deficit, suggesting a dose-dependent neurotoxic effect of lead on the developing olfactory system. Co-treatment with BHA, a known antioxidant, partially mitigated these deficits, indicating that oxidative stress may play a central role in lead-induced olfactory dysfunction. These findings suggest that lead acetate disrupts olfactory signaling pathways in larvae, likely through oxidative mechanisms, and that antioxidants like BHA could offer a protective strategy against lead neurotoxicity. Our results show that the olfactory in third-instar *Drosophila* larvae are dramatically decreased by chronic exposure to lead acetate in a time- and dose-dependent manner. This is the first report on the toxic effects of lead on fruit fly larvae's cognitive abilities. To fully comprehend the molecular causes of lead-induced reduced cognitive abilities in larvae, more investigations are needed.

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