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Effect of fluoride on the learning and memory ability of larvae of *Zaprionus indianus*



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Abstract

Background: Many pesticides contain fluoride that enters the food chain and affect the non-target organisms. Fluoride is a known neurotoxin and may cause neurobehavioral defects. A study was conducted to see the effect of fluoride on the learning and memory ability of larvae of *Zaprionus indianus*. The learning and memory ability of 2nd instar larvae of normal (control) and sodium fluoride (NaF)-treated *Zaprionus indianus* was compared.

Results: Sublethal concentration of NaF for *Z. indianus* was found to be 0.8 ppm. Olfactory assay results showed that the larvae of normal (control) *Z. indianus* had better learning and memory ability in comparison to NaF-treated larvae.

Conclusions: This study indicates that the insects exposed to pesticides containing fluoride may have difficulty in locating food sources and carrying out pollination.

Keywords: *Zaprionus indianus*, Sodium fluoride, Olfactory assay, 2nd instar larvae, Learning and memory

Background

Exposure to fluoride can occur through dietary intake, respiration, and water. Fluoride enters the environment through volcanic eruptions, rock dissolution, and numerous human activities (coal burning, ore processing, production and use of fertilizers, and industrial plants). Many pesticides, insecticides, and weedicides contain fluoride in high concentrations, and the overuse of such chemicals paves way for fluoride to enter the system of non-targeted organisms such as human beings and other animals and cause derogatory effects. Acute pesticide poisoning occurs frequently in children worldwide, and subclinical pesticide toxicity is also widespread (Grandjean & Landrigan, 2014). Clinical data suggest that acute pesticide poisoning during childhood might lead to lasting neurobehavioral deficits (Kofman, Berger, Massarwa, Friedman, & Jaffar, 2006; London et al., 2012). Thus, there is a need to study the neurotoxic effect of fluoride.

Fruit flies and mammals share many genes suggesting that the molecular mechanisms of behavioral plasticity might also be shared (Rubin et al. 2000). Short life span, large number of offspring produced, a well-known anatomy, and occurrence of a wide variety of mutants are convenient characteristics of fruit flies as a model organism (Jeibman and Paulus, 2009).

Olfaction in fruit fly is crucial for a variety of behaviors, including associative learning (Quinn et al., 1974, Tully and Quinn, 1985) courtship (Gailley et al. 1986), foraging (Shaver et al. 1998; Frye and Dickinson, 2004), and flight (Schneiderman and Trimarchi, 1995). Fruit flies can learn to associate olfactory or visual cues with rewarding or punishing reinforcement. Fruit fly memory persists for hours or days, depending on the training protocol. Multiple spaced training trials form long-term memory that can persist for days (Keene and Waddell, 2007). Fruit fly larvae can be used as model organisms to study the neurotoxic effect of fluoride.

Zaprionus indianus (Gupta, 1970) is an arthropod belonging to the fruit fly family Drosophilidae. *Z. indianus*

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Table 1 Olfactory Response Index (ORI) of 2nd instar larvae of *Zaprionus indianus* for different concentrations of iso-amyl acetate (IAA) and apple cider vinegar (ACV)

Different concentration of IAA	Olfactory Response Index (ORI)	Mean \pm SE	Different concentration of ACV	Olfactory Response Index (ORI)	Mean \pm SE
Distilled water (DW)	0.00, 0.04, 0.00	0.01 \pm 0.01	Distilled water (DW)	0.00, - 0.02, 0.00	- 0.01 \pm 0.01
10 ⁻¹	0.03, - 0.05, 0.16	0.05 \pm 0.06	10 ⁻¹	0.36, 0.14, 0.08	0.19 \pm 0.09
10 ⁻²	- 0.19, - 0.08, - 0.21	- 0.16 \pm 0.04	10 ⁻²	0.07, 0.25, 0.25	0.19 \pm 0.06
10 ⁻⁴	0.05, 0.02, 0.32	0.13 \pm 0.09	10 ⁻⁴	- 0.20, 0.03, 0.06	- 0.04 \pm 0.08

is abundantly found around fruit trees such as guava and mango and may help the trees in carrying out the process of pollination. Due to this, *Z. indianus* is at a risk of being exposed to insecticides that contain fluoride that is a potential neurotoxin. This may result in the organism losing track of its trail, and if that happens, the fly will ultimately die because it will not be able to find its food, and the trees dependent on the fly for the dispersal of pollen may suffer too. A study on this aspect has not been conducted so far. This paper presents the effect of sublethal level of sodium fluoride (NaF) on the learning and memory ability of 2nd instar larvae of *Z. indianus* (Gupta, 1970).

Methods

The assessment of memory ability of *Z. indianus* was done using its 2nd instar larvae. *Z. indianus* flies were trapped using fruit baits and cultured in the laboratory on cornmeal medium. Single line culture of flies was maintained by transferring a gravid fly in separate cornmeal medium containing bottles. The larvae obtained were assessed for their learning and memory ability with the help of olfactory assay following Scherer, Stocker, and Bertram Gerber (2003). Four sets of 100 ml cornmeal medium were prepared to be poured in sixteen glass bottles. Each set contained four bottles. Out of all the four sets, one set was used as control, and the rest three were experimental set up.

First, iso-amyl acetate (IAA) was used as attractant since *Drosophila melanogaster* is attracted towards it (Khurana and Siddiqui, 2013). But *Z. indianus* larvae did not show appreciable response towards IAA. Next, apple cider vinegar (ACV) was used as odourant to attract *Zaprionus indianus* larvae following Joshi, Biddinger, Demchak, and Deppen (2014). Olfaction assay was performed to determine the concentration at which larvae of *Z. indianus* was maximum attracted. Olfactory assay was performed with following concentrations ACV, i.e., 10⁻¹, 10⁻², and 10⁻⁴.

Thereafter, 1000 ppm NaF stock solution was prepared by adding 2.21 g of NaF into 1000 ml distilled water. NaF of the concentrations 0.8 ppm, 1.0 ppm, and 1.5 ppm was taken to treat the flies. Three sets of cornmeal medium were prepared. Each set contained four bottles.

The first set had 0.8 ppm NaF, the second set had 1.0 ppm NaF, and the third set had 1.5 ppm NaF containing cornmeal medium. *Zaprionus indianus* flies from single line stock culture were transferred into each of these bottles such that each bottle contained at least one gravid fly.

Olfactory assay

Plain agar petri plates were taken. A filter paper was taken on which a circle and two vertical lines were drawn in the center, and two diametrically opposite points were marked close to the periphery of the petri plates and were termed as C₁ and C₂.

Procedure for olfactory assay

Control test

Agar plate was divided into two halves, and one drop of distilled water was placed on each side with the help of a dropper. Sixty 2nd instar larvae were introduced at the center, covered with black box and left for 2 min. After 2 min, larvae were counted on both sides (C₁ and C₂), and Olfactory Response Index (ORI) was calculated.

Experimental test

10⁻² concentration of ACV was used as attractant on one side and distilled water on the other side of petri plate with plain agar. Same set of 2nd instar larvae was introduced at the center covered with black box and left

Table 2 Culture of flies in 0.8 ppm NaF in four different bottles

Date	A	B	C	D
13.09.18	5 flies	5 flies	2 flies	5 flies
14.09.18	5 flies + eggs	5 flies + eggs	2 flies + eggs	4 flies + eggs
15.09.18	5 flies + larvae	4 flies + larvae	2 flies + larvae	4 flies + larvae
16.09.18	5 flies + larvae	4 flies + larvae	2 flies + larvae	4 flies + larvae
17.09.18	5 flies + pupa	4 flies + pupa	2 flies + pupa	4 flies + pupa
18.09.18	5 flies + pupa	3 flies + pupa	2 flies + pupa	4 flies + pupa
19.09.18	5 flies + pupa	3 flies + pupa	2 flies + pupa	4 flies + pupa
20.09.18	20 flies	3 flies + pupa	7 flies	10 flies
21.09.18	20 flies	10 flies	7 flies	10 flies
22.09.18	20 flies	10 flies	7 flies	10 flies

Table 3 Culture of flies in 1.0 ppm NaF in four different bottles

Date	A	B	C	D
13.09.18	5 flies	5 flies	5 flies	3 flies
14.09.18	5 flies	5 flies + eggs	4 flies + eggs	3 flies
15.09.18	5 flies	4 flies + eggs	3 flies + eggs	2 flies
16.09.18	4 flies	4 flies, no larvae	3 flies, no larvae	Dead flies
17.09.18	4 flies	3 flies, no larvae	2 flies, no larvae	Dead flies
18.09.18	2 flies	3 flies, no larvae	Dead flies	Dead flies
19.09.18	2 flies	Dead flies	Dead flies	Dead flies

for 2 min. After 2 min, larvae were counted on both sides, and ORI was calculated.

Formula for calculating ORI

$$\text{ORI} = C_1 - C_2 / C_1 + C_2$$

Here,

C_1 = no. of larvae on side one (ACV)

C_2 = no. of larvae on side second (distilled water)

Avoidance test

Next, agar plate was neatly cut into half, and half of it was removed and replaced with agar containing 20 mM NaCl. NaCl played the role of irritant. ACV was placed on the side containing NaCl, and on the other side distilled water was put. Same set of larvae was introduced in the center, and assay was performed as during experimental test. ORI was calculated.

Confirmatory test

Next, same set of larvae was again placed on plain agar petri plate containing ACV on one side and distilled water on the other, and olfactory assay performed as during experimental test. ORI is calculated.

Experimental set up

Similarly, larvae treated with sublethal level of NaF were also introduced on plain agar petri plates and control test, experimental test, avoidance test, and confirmatory test were performed.

Table 4 Culture of flies in 1.5 ppm NaF in four different bottles

Date	A	B	C	D
13.09.18	3 flies	4 flies	6 flies	4 flies
14.09.18	3 flies + no eggs	Dead flies	2 flies	1 fly
15.09.18	2 flies, no larvae	Dead flies	2 flies	Dead flies
16.09.18	Dead flies	Dead flies	Dead flies	Dead flies
17.09.18	Dead flies	Dead flies	Dead flies	Dead flies
18.09.18	Dead flies	Dead flies	Dead flies	Dead flies
19.09.18	Dead flies	Dead flies	Dead flies	Dead flies

Table 5 Olfactory Response Index (ORI) for normal *Z. indianus* larvae

Larvae	DW vs DW	ACV vs DW	Avoidance test	Confirmatory test
1st set	0	0.34	0.11	- 0.08
2nd set	0.01	0.26	0.14	- 0.07
3rd set	0	0.16	0.19	- 0.03

Statistical analysis

Students *t*-test was performed to compare the mean ORI of control vs NaF-treated flies, and $P < 0.05$ was considered as statistically significant.

Results

The ORI of *Z. indianus* larvae towards IAA and ACV is shown in Table 1. The larvae were not found to be attracted towards IAA. However, they showed attraction towards ACV. 10^{-2} was the favored concentration. So, 10^{-2} concentration of ACV was taken for further olfactory assay.

Sublethal concentration of NaF for *Z. indianus* was found to be 0.8 ppm. This concentration was used for performing olfactory assay because flies were found to survive and reproduce in this concentration (Table 2). On the other hand, 1.0 ppm and 1.5 ppm NaF concentrations were found to be lethal for the flies as at this concentration the flies were unable to reproduce and grow in number (Tables 3 and 4).

Larvae reared on normal cornmeal medium were taken as control and were assessed for their learning and memory ability by performing olfactory assay with 10^{-2} concentration of ACV (Table 5). *Z. indianus* larvae reared on 0.8 ppm concentration of NaF were assessed for their learning and memory ability by performing olfactory assay with 10^{-2} concentration of apple cider vinegar (Table 6). A statistically significant difference was found in the means of ORI of normal vs NaF-treated larvae during the confirmatory test (t , 4.3; $df = 4$; $P < 0.05$) (Table 7).

Discussion

Olfactory assay of the larvae of native *Z. indianus* has been conducted for the first time in the present study. Khurana and Siddiqui (2013) studied the response of 3rd instar *Drosophila* larvae towards 53 odorants. Such

Table 6 Olfactory Response Index (ORI) for NaF-treated *Zaprionus indianus* larvae

Larvae	DW versus DW	ACV versus DW	Avoidance test	Confirmatory test
1st set	0.02	0.10	0.13	0.02
2nd set	0	0.21	0.17	0.07
3rd set	0.01	0.13	0.14	0.01

Table 7 Comparison of Olfactory Response Index of 2nd instar larvae of normal and fluoride-treated *Zaprionus indianus*

	Control (normal)	Mean ± SE	NaF treated	Mean ± SE
Control	0, 0.01, 0	0.00 ± 0.003	0.02, 0.12, 0.03	0.05 ± 0.03
Experimental	0.06, 0.26, 0.16	0.16 ± 0.05	0.19, 0.34, 0.13	0.22 ± 0.06
Avoidance	0.11, 0.14, 0.23	0.33 ± 0.03	0.09, 0.10, - 0.17	0.01 ± 0.08
Confirmatory	- 0.08, - 0.07, - 0.03	- 0.06 ± 0.01*	0.02, 0, 0.01	0.01 ± 0.005*

*Significant difference at $P < 0.05$

elaborate studies on response profile of *Drosophila* larvae were very valuable while performing olfactory assay. Tabassum, Kumari, Singh, and Yasmin (2017) studied a comparative account of the olfactory behavior of pure-line *Drosophila melanogaster* (inbred up to 10 generations) and CsBz with that of native *Drosophila melanogaster* by using iso-amyl acetate odourant.

Zaprionus indianus larvae did not show appreciable response towards iso-amyl acetate. So, based on experiments done by Joshi et al. (2014) at Pennsylvania, ACV was used as attractant. Based on the ORI values obtained, it was observed that *Z. indianus* larvae showed maximum attraction at 10^{-2} concentration apple cider vinegar.

In the experiment, it was found that 2nd instar larvae of *Zaprionus indianus* showed abnormalities on treatment with NaF. At concentration of NaF, more than 0.8 ppm (i.e., 1.0 ppm and 1.5 ppm) *Z. indianus* flies did not lay eggs, and flies died in a few days. Due to the effect of fluoride, the learning and memory ability of *Zaprionus* larvae was hampered, which became evident with the ORI results obtained (positive value of confirmatory test), as opposed to the ORI results of normal larvae (not exposed to NaF), where ORI value was negative during confirmatory test. The abnormalities displayed by NaF-treated *Zaprionus indianus* larvae can be said to be because of NaF reacting with the brain of the larvae. Fluoride is a known neurotoxin (Spittle, 2011). F toxicity may also result in low IQ children (Yasmin et al. 2013).

Though, the killing action of fluoride can be very helpful in insecticides (Metcalf, 1966), the aspect of fluoride affecting the nervous system cannot be dealt leniently (Grandjean and Landrigan, 2014). *Z. indianus* has been considered as a pest in many countries such as Veracruz in Mexico (Lasa and Tadeo, 2015). But the fact that it is found in the orchards indicates that it may be helping in the process of pollination (Landolt et al., 2012). This makes the fly a significant component of the natural ecosystem. If all such flies and other insects are treated with pesticides containing fluoride, it can lead to their death or reduced efficiency in carrying out pollination. In either case, the whole system of symbiotic association between trees and the insects will be disrupted. This will ultimately lead to reduced productivity of the trees.

Conclusions

The study showed that NaF-treated larvae suffered some neurological disorder that affected their learning and memory ability. Pesticides containing fluoride can cause death of non-target insect populations or can reduce their efficiency in carrying out pollination by affecting their learning and memory aspects.

Abbreviations

NaF: Sodium fluoride; IAA: Iso-amyl acetate; ACV: Apple cider vinegar; ORI: Olfactory Response Index

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Authors' contributions

Equally contributed. The author(s) read and approved the final manuscript.

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