IRIS
Journal for Young Scientists
ISSN 2278 – 618X (Print)
ISSN 2278 – 6384 (Online)

http://www.patnawomenscollege.in/journal

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

• **Diksha Mishra** • **Rishu Kumari** • **Smita Ranjan**
• **Shahla Yasmin**

Received : November 2018
Accepted : March 2019
Corresponding Author : Shahla Yasmin

Abstract : The 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 control (normal) and Sodium Fluoride (NaF) treated *Zaprionus indianus* was compared. For this study, four olfactory assay setup was designed namely, control, experimental, avoidance and confirmatory. Sixty larvae of the same age group were used for each assay. Result showed that the larvae of control (normal) *Z. indianus* had better learning and memory ability in comparison to NaF treated *Z. indianus* larvae.

Keywords*: Z. indianus, Sodium Fluoride, Olfactory assay, 2ndinstar larvae.*

Diksha Mishra
B.Sc. III year, Zoology (Hons.),
Session : 2016-2019, Patna Women's College,
Patna University, Patna, Bihar, India

Rishu Kumari
B.Sc. III year, Zoology (Hons.),
Session : 2016-2019, Patna Women's College,
Patna University, Patna, Bihar, India

Smita Ranjan
B.Sc. III year, Zoology (Hons.),
Session : 2016-2019, Patna Women's College,
Patna University, Patna, Bihar, India

Shahla Yasmin
Head, Department of Zoology, Patna Women's College,
Bailey Road, Patna–800 001, Bihar, India
E-mail : shahla_apex@yahoo.co.in

Introduction:

Exposure to fluoride can occur through dietary intake, respiration and water. It 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 and Landrigan,2014). Clinical data suggest that acute pesticide poisoning during childhood might lead to neurobehavioural deficits. (Kolman et al, 2006; London et al, 2012). Thus, there is a need to verify the neurotoxic effect of fluoride.

To establish fluoride as a neurotoxin, fruitfly can be used as a model organism. Modelling human brain diseases in fruitflies offers several advantages for investigation of molecular and cellular mechanisms underlying human diseases. Short life span, large number of offspring produced, a well known anatomy and occurrence of a wide variety of mutants are convenient characteristics of

fruitflies as a model organism (Jeibman and Paulus, 2009).

Zaprionus indianus, an arthropod belonging to the fruitfly family Drosophilidae (Gupta, 1970) was employed for this research because it was easily available in the college campus.

Zaprionus indianus are 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 won't be able to find its food, and the trees dependent on the fly for the dispersal of pollen will suffer too. A study on this aspect has not been conducted so far. This paper presents the effect of sub-lethal level of Sodium Fluoride (NaF) on the learning and memory ability of 2nd instar larvae of *Z. indianus.*

Materials and Methods:

The assessment of memory ability of *Z. indianus* was done using its 2nd instar larvae. *Z. indianus* flies, trapped using fruit baits were cultured in laboratory on cornmeal medium. Single line culture of flies was maintained by transferring a gravid fly in separate cornmeal medium containing bottles. The larvae so obtained were assessed for their learning and memory ability with the help of olfactory assay following Scherer et al (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 rest three were experimental setup.

Determination of suitable attractant for *Zaprionus* larvae : First of all Iso Amyl acetate (IAA) was used as attractant since *Drosophila melanogaster* is attracted towards it (Khurana and Siddiqui, 2013) because it's smell is similar to that of banana. But *Z. indianus* larvae did not show appreciable response towards IAA. Next, Apple Cider Vinegar (ACV) was used to attract *Z. indianus* larvae following Joshi et al (2014). Olfaction assay was performed to determine the concentration at which larvae of *Z. indianus* was maximum attracted.

Preparation of different concentrations of apple cider vinegar : Apple Cider Vinegar (ACV) was used as odourant to attract *Z. indianus* larvae. ACV was diluted in concentrations in the following concentrations by serial dilution method:10^{-1},10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}.

Preparation of 1000ppm NaF stock solution : 2.21 grams NaF was added to 1000ml distilled water to give 1000ppm NaF solution.

NaF of the concentrations 0.8ppm, 1.0 ppm and 1.5 ppm was taken to treat the flies.

Preparation of fluoride containing cornmeal medium

(i) For preparing 0.8ppm NaF medium, 100ml cornmeal medium contents + 99.2ml distilled water + 0.8 ml NaF solution were taken.

(ii) For preparing 1.0 ppm NaF medium, 100ml cornmeal medium contents + 99ml distilled water + 1ml NaF solution were taken.

(iii) For preparing 1.5 ppm NaF medium, 100ml cornmeal medium contents + 98.5ml distilled water + 1.5ml NaF solution were taken.

Three sets of cornmeal medium were prepared. Each set contained four bottles. The first set had 0.8ppm NaF, the second set had 1.0ppm NaF and the third set had 1.5ppm 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. The survival and reproduction of flies were monitored.

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 centre, and two points were marked along the diameter close to the periphery, which were termed C1 and C2 (Fig. 1).

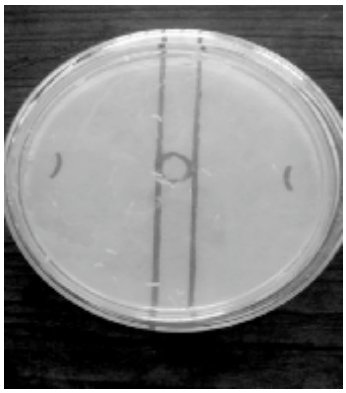

Fig. 1. A set up for olfactory assay

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 two minutes. After two minutes, larvae were counted on both sides (C1 and C2) and 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 were introduced at the center, covered with black box and left for two minutes. After two minutes, larvae were counted on both sides and ORI was calculated.

Formula for calculating ORI :

ORI=C1-C2/C1+C2

Here,

C1= no. of larvae on side one (ACV)

C2=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 20mM 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 centre and assay was performed as during experimental test. ORI was calculated.

Confirmatory test: Next, same set of larvae were 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 was calculated.

Experimental set up: Similarly, larvae were obtained from fly culture bottles treated with NaF and olfactory assay performed. Larvae treated with sub lethal level of Fluoride concentration were introduced on plain agar petri plates and control test, experimental test, avoidance test and confirmatory test were performed.

Statistical analysis: Students t-test was performed to compare the mean ORI of control versus NaF treated flies and $P<0.05$ was considered as statistically significant.

Results and Discussion :

Life cycle of *Z. indianus* was observed to be completed in an average 9 (8 to 10) days at temperature = 30–32°C (Table 1). This is comparable to the life cycle of *Drosophila melanogaster* which is also completed in 9 days (Tabassum et al, 2017; Singh et al,2018). Eggs appeared a day after transferring a gravid fly into cornmeal medium containing bottle. 1st instar larvae were observed the following day, which were seen moving inside the medium. On the fourth day, 2nd instar larvae were observed. On fifth day, 3rd instar larvae were observed, which were observed to climb up the walls of the bottle. The following day (day six), the less active 3rd instar larvae got transformed into the stationary Pupa stage, which remained in pupa stage for next 3 days. Next, flies ecloded from pupa. The various stages of life cycle of *Z. indianus* has been summarized in Table 1.

Table 1. Stages during the life cycle of *Z. indianus*

S. No.	Date	Observation	Temp	Humidity
1.	6.03.18	Single gravid fly transferred incornmeal medium containing bottle	30° C	29%
2.	7.03.18	Eggs observed	31°C	33%
3.	8.03.18	1st instar larvae observed in the medium	30°C	33%
4.	9.03.18	2nd instar larvae observed in theMedium	30°C	35%
5.	10.03.18	3rd instar larvae observed climbingup the walls of the cornmeal bottle	32°C	36%
6.	11.03.18	Pupae observed. 3rd instar larvae also present	34°C	24%
7.	12.03.18	Pupae observed	32°C	36%
8.	13.03.18	Pupae observed	32°C	36%
9.	14.03.18	Flies eclode. Total 4 flies observed	32°C	43%

The Olfactory Response Index (ORI) of *Z. indianus* larvae towards Iso Amyl Acetate (IAA) is shown in Table 2. The larvae were not found to be attracted towards Iso Amyl Acetate(IAA). However, they showed attraction towards Apple Cider Vinegar (ACV) (Table 3). 10^{-2} was the most favoured concentration. So, 10^{-2} concentration of ACV was taken for further olfactory assay.

Table 2. Olfactory response index (ORI) of 2nd instar larvae of *Zaprionus indianus* for different concentrations of Iso Amyl Acetate (IAA)

DIFFERENT CONCENTRATION OF IAA	ORI	MEAN	SD	SE
DW	0.00, 0.04,0.00	0.01	0.02	0.01
10^{-1}	0.03, -0.05, 0.16	0.05	0.11	0.06
10^{-2}	-0.19, -0.08, -0.21	-0.16	0.07	0.04
10^{-6}	0.05, 0.02, 0.32	0.13	0.16	0.09

*SD= Standard deviation

*SE=Standard error

Table 3. Olfactory response index (ORI) of 2nd instar larvae of *Zaprionus indianus* for different concentrations of Apple Cider Vinegar (ACV)

DIFFERENT CONCENTRATION OF IAA	ORI	MEAN	SD	SE
DW	0.00, -0.02, 0.00	-0.01	0.01	0.01
10^{-1}	0.36, 0.14, 0.08	0.19	0.15	0.09
10^{-2}	0.07,0.25,0.25	0.19	0.10	0.06
10^{-4}	-0.20, 0.03, 0.06	-0.04	0.14	0.08
10^{-6}	-0.18, 0.27, 0.15	0.08	0.23	0.13

*SD= Standard deviation

*SE=Standard error

Sub lethal concentration of NaF for *Z. indianus* was found to be 0.8ppm. This concentration was used for performing olfactory assay because flies were found to survive and reproduce in this concentration (Table 4).On the other hand 1.0 ppm and 1.5 ppm NaF concentration were found to be lethal for the flies as at this concentration the flies were unable to reproduce and grow in number (Tables 5 and 6).

Determination of sub lethal level of fluoride concentration for *Z. indianus*:

Table 4. Culture of flies in 0.8 ppm NaF

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 5. Culture of flies in 1.0 ppm NaF

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	–
17.09.18	4 flies	3 flies, no larvae	2 flies, no larvae	–
18.09.18	2 flies	3 flies, no larvae	–	–
19.09.18	2 flies	–	–	–

Table 6. Culture of flies in 1.5 ppm NaF

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

Larvae reared on normal cornmeal medium were taken as control (Table 7). *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 8). A comparative account of both the memory assays is shown in Table 9. A statistically significant difference was found in the means of ORI of normal versus NaF treated larvae during the confirmatory test (t-4.3, df=4, P<0.05) (Table 9).

The comparative account of learning and memory in normal vs fluoride treated *Z. indianus* larvae is given below:

Table 7. Olfactory Response Index (ORI) for normal *Z. aprionus indianus*

LARVAE	CONTROL	ACV vs DW	AVOIDANCE TEST	CONFIRMATION 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

Table 8. Olfactory Response Index (ORI) for NaF treated *Zaprionus indianus*

LARVAE	DW vs DW	ACV vs DW	AVOIDANCE TEST	CONFIRMATION 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 9. Comparison of olfactory response index of 2nd instar larvae of normal and fluoride treated *Zaprionus indianus*:

	NORMAL	MEAN	SD	SE	NaF TREATED	MEAN	SD	SE
CONTROL	0, 0.01, 0	0.00	0.005	0.003	0.02,0.12,0.03	0.05	0.05	0.03
EXPERIMENTAL	0.06,0.26,0.16	0.16	0.09	0.05	0.19,0.34, 0.13	0.22	0.11	0.06
AVOIDANCE	0.11,0.14,0.23	0.33	0.06	0.03	0.09, 0.10, -0.17	0.01	0.01	0.08
CONFIRMATORY	-0.08, -0.07, -0.03	-0.06*	0.02	0.01	0.02,0,0.01	0.01*	0.01	0.005

*Significant difference at p<0.05

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. Fruitfly 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).

Khurana and Siddiqui (2013) studied the response of 3rd instar *Drosophila* larvae towards 53 odourants. Such elaborate studies on response profile of *Drosophila* larvae were very valuable while performing olfactory assay. Tabassum et al (2017) studied a comparative account of the olfactory behavior of pureline *Drosophila melanogaster* (inbred upto 10 generations) and CsBz with that of native *Drosophila melanogaster* by using Iso-Amyl Acetate odourant. Olfactory assay of the larvae of native *Z. indianus* has been conducted for the first time in the present study.

Zaprionus indianus larvae did not show appreciable response towards Iso Amyl Acetate. So, based on experiments done by Joshi et al., (2014) at Pennysylvania, Apple Cider Vinegar (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. Also, another point that was noted while performing olfaction assay of *Z. indianus* with ACV was the fact that Z. indianus larvae showed strikingly higher response for 10^{-6} concentration of ACV after a downfall of negative ORI value at 10^{-4} concentration of ACV. This indicated selective response of *Z. indianus* larvae towards different concentrations of ACV. Similar finding was reported by Khurana and Siddiqui (2013) in *D. melanogaster.*

In the experiment, it was found that, 2nd instar larvae of *Zaprionus indianus* showed abnormalities on treatment with Sodium Fluoride. 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. It is proposed that this abnormality is attributed to the exposure of the organism with fluoride. 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 known as a neurotoxin (Spittle, 2018). Comparing the ORI values obtained in each set, it was evident that NaF treated larvae did suffer some neurological disorder that affected its learning and memory ability.

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 (Adam et al, 2015). 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:

From the results obtained, it can be concluded that *Z. indianus* larvae are maximum attracted towards 10-2 concentration of ACV. The lethal level of NaF concentration for *Z. indianus* larvae was 1.0ppm. The sub-lethal level was identified to be 0.8ppm NaF. Further it was identified that the learning and memory capacity of normal *Z. indianus* larvae was better than those treated with

NaF. The primary cause of this atypical behavior of NaF treated *Z. indianus* may be due to interference of fluoride with the activity of nerves of the brain of larvae.

Acknowledgements:

We are grateful to Dr. Sister M. Rashmi A. C., Principal, Patna Women's College for providing us all the facilities for carrying out the study. We are also grateful to Dr. M. C. Arunan of HBCSE, Mumbai and CUBE mailing group for their valuable suggestions. We are also thankful to the Research Committee for providing the facilities.

References:

Frye M. A., Dickinson M. H. (2004). Motor output reflects the linear superposition of visual and olfactory inputs in *Drosophila*. *Journal of Experimental Biology*. 207:123–131.

Gailey D. A., Lacaillade R. C., Hall J. C. (1986). Chemosensory elements of courtship in normal and mutant, olfaction- deficient *Drosophila melanogaster*. *Behavioural Genetics*. 16:375– 405.

Grandjean P., Landrigan P. (2014). Neurobehvioural effects of developmental toxicity. *Lancet Neurology* 13(3):330-338

Gupta, J. P. (1970). Description of a new species of *Phorticella Zaprionus* (Drosophilidae) from India *Proceedings Indian National Science Academy*. 36:62–70.

Jeibmann A., Paulus W. (2009). *Drosophila melanogaster* as a model organism for brain diseases. *International Journal of Molecular Sciences*. 10 (2) 407-440.

Joshi N.K., Biddinger D.J., Demchak K., Deppen A. (2014). First report of *Zaprionus indianus* in Pennysylvania. *Journal of Insect Sciences*. 14:259-263.

Keene A. C., Waddell S. (2007). *Drosophila* olfactory memory: single genes to complex neural circuits. *Nature Reviews Neuroscience* (8):341–354.

Khurana S., Siddiqui O. (2013). Olfactory Responses of *Drosophila* Larvae. *Chemical senses*. 38:315-323.

Kofman O., Berger A., Massarwa A., Friedman A., Jaffar A.A., Massarwa A. (2006). Motor inhibition and learning impairments in school-aged children following exposure to organophosphate pesticides in infancy. Journal of Pediatric Research. 60: 88-92

Landolt PJ., Adams T., Davis T. S., Rogg H. (2012). Spotted Wing *Drosophila*, *Drosophila suzukii* (Diptera: Drosophilidae), Trapped with Combinations of Wines and Vinegars. *Florida Entomologist*. 95(2):326-332.

Lasa R., Tadeo E. (2015). Invasive Drosophilid Pests *Drosophila suzukii* and *Zaprionus indianus* (Diptera: Drosophilidae) in Veracruz, Mexico. *Florida Entomologist*. 98(3): 987–988.

London L., Beseler C., Beseler C., Bouchard M.F. (2012). Neurobehavioral and neurodevelopmental effects of pesticide exposures. *Neurotoxicology* 33: 887-896

Metcalf R. L. (2015). Fluorine-containing insecticides. In smith F.A. (eds.) Pharmacology of fluorides. Hand book of Experimental Pharmacology. 20:355-386.

Quinn W. G., Benzer S., Harris W. A. *(*1974*).* Conditioned behavior in *Drosophila melanogaster. Proceedings of National Academy of Sciences USA.* 71*:*708–712*.*

Scherer S., Stocker R., Gerber B., (2003). Olfactory Learning in Individually Assayed *Drosophila* Larvae. *Learning memory*. 10(3):217-225

Schneiderman A. M., Trimarchi J. R. (1995). Different neural pathways coordinate *Drosophila* flight initiations evoked by visual and olfactory stimuli. *Journal of Experimental Biology* 198:1099– 1104.

Shaver S.A., Varnam C.J., Hilliker A.J., Sokolowski M.B., (1998). The foraging gene affects adult but not larval olfactory-related behavior in *Drosophila melanogaster*. *Behavioural Brain Research*. 95:23– 29.

Singh V., Choudhary R., Shah S. (2018). Amelioration of Fluoride toxicity on the reproductive output of *Drosophila melanogaster* with L. Ascorbic acid. *IRIS, Journal for Young Scientist*. 8-6.

Spittle B. (2011). Neurotoxic effects of fluoride. *Fluoride* 44 (3):117-124

Tabassum F., Kumari N., Singh R. (2017). Olfactory behavior and learning in native versus Lab-bred *Drosophila melanogaster. IRIS Journal for Young Scientists*. (7):7-13.

Tully T., Quinn W. G. *(*1985). Classical conditioning and retention in normal and mutant *Drosophila melanogaster. Journal Comp Physiology*. 157*:*263–277.