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SODIUM FLUORIDE DISTRESSES CHROMATOPHORE INTEGRITY AND BEHAVIOUR OF GUPPY, *POECILIA RETICULATA PETERS*

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ABSTRACT The present study was undertaken to investigate the toxicological effect of fluoride (as NaF) on chromatophores in skin of larvivorous fish, *Poecilia reticulata* Peters after chronic exposure. Guppy is supposed to be amongst the most colour-polymorphic vertebrates in the animal kingdom. Fish were exposed to three sub-lethal concentrations of Sodium fluoride viz. lowest (5.75 ppm), lower intermediate (7.18 ppm) and higher intermediate (9.58 ppm), which were selected on the basis of 96 hrs. LC_{50} value of 115 ppm. Anomalous behavioral alterations observed in NaF intoxicated fish were restlessness, erratic swimming movement, periodic surface to bottom movement, recurrent surfacing activity, loss of equilibrium, amplified gulping and increased opercular activity were pragmatically seen. Significant variation in colouration was observed in all the experimental groups as compared to control. The melanophores showed notable pigment aggregation state and/or pigment dispersion state. Structurally, melanophores were distinguished into three types viz., reticulate, stellate and punctuate. The severity in distortion of structure and number of chromatophore increased with increasing concentrations of fluoride. Several distortions in morphology and even functional integrity of chromatophores when compared with the control group were observed, for instance, rupture of pigment cells accompanied by breakage in dendritic processes, conversion of one type of chromatophore into another type, augmentation and detachment between adjacent chromatophores, variation in number with complete loss of cellular activity. The derangement in structural integrity was observed to be dose dependent and increased with increasing concentrations of Sodium fluoride, thus making the fish unfit for survival. The attractive pigmentation of the male Guppy fish was abridged making them less attractive for their mate thus affecting reproductive potential of the fish.

KEYWORDS : *Poecilia reticulata* Peters, Sodium fluoride, Behaviour, Chromatophore.

INTRODUCTION:

Animal model selected for the present investigation is larvivorous fish, *Poecilia reticulata* Peters, commonly known as 'Guppy'. Apart from widely being used in the biological control of mosquitoes, Guppy fish often find place in freshwater aquaria in houses, offices and places of recreation due to their bizarre and vivid colouration. Aquarium dealers often culture and rear Guppy, as it forms very economical live food for a variety of comparatively larger carnivorous fishes of the aquaria. Guppy is a sturdy, prolific, livebearer fish with a minimal maintenance and hence favoured over other livebearer larvivorous fishes. Nature has given them brilliantly coloured patterns that would make even a peacock jealous. No two males appear to have exactly the identical designs and it is this extreme variability of colour and finnage that is responsible for their selective breeding. In Guppy fish skin contains numerous colour cells known as chromatophores which control the colour and pattern of the fish. These colour cells are governed by the genetic characters which in turn controls colour in the fish. They contain varying amount of melanin which affect the skin colour background. The body colours of both females and males were determined mainly by the existence of several types of chromatophores and the genetic polymorphisms of formation of these chromatophores have been described (Ueshima et al., 1998). Farha Aziz et al. (2013) observed alteration in colour of the skin that changed from normal dark pigmentation to relatively lighter colour on the dorsal and lateral part after sixth day of exposure under fluoridation in edible fish *Notopterus notopterus*.

Chromatophores are pigment containing and light reflecting cells present in the skin of various vertebrates like fishes, amphibians, reptiles and mammals (Zarnescu, 2007). In Vertebrates, chromatophores are mostly classified into five categories according to Fujii (2000). These are the melanophore (black), erythrophore (red), xanthophore (yellow), leucophore (white) and iridophore (reflecting) and also recently reported the blue chromatophore (cyanophores). No chromatophores which have fluorescence have been reported, however, several pigments which have fluorescence have been reported, such as pteridines. The genetic control on formation of chromatophores, melanophore, xanthophore and erythrophore, has been observed in the Guppy as a body colour polymorphism (Ueshima et al., 1998). Most pigment-containing chromatophores are not only specialized in producing and storing, but also in translocating their numerous pigment particles (Fujii, 2000). Chromatophores plays a significant role and are the fundamental system for physiological control over body colouration. Lightening and darkening of fish skin occurs due to aggregation and dispersal of pigment. The accretion of pigment granules, melanophores to the centre of the cell consequences in skin lightening whereas dispersal of pigment all over the cells results in darkening.

Fluoride is an effective agent for the prevention of dental caries. However the mechanism of how excessive fluoride exposure causes fluorosis remains ambiguous. This compound is, however, highly poisonous and causes teratogenic effects. Wang et al. (2009) and Sharma et al. (2007) found that fluoride exposure disrupts the synthesis of collagen and leads to its breakdown in bone, tendon, muscle, skin, cartilage, lung, kidney and trachea. Ultrastructural changes in vital tissue like liver of *Poecilia reticulata* Peters induced by fluoride have also been reported (Hitesh Shingadia, 2015). The transformation in chromatophore configuration is controlled by the nervous and endocrine systems (Hoar, 1987; Nagabhusnam and Sarojini, 1989 and Tripathi et al., 2005). Environmental information is processed in the CNS and transmitted to the melanophores, where both the hormonal and neuronal regulations result in appropriate chromatic reactions (Pradeep et al., 2007). Apart from this, numerous ecological factors such as light, water quality, temperature, salinity and pollutants are also responsible to upset colour change (Fujii, 1969; Watanabe et al., 1965; Tripathi et al., 2005 and Pradeep et al., 2007). There are various reports indicating adverse effect of pollutants in fishes but not much information is available concerning their effect on fish pigmentation. Since fluoride has been reported to affect nervous as well as endocrine systems in animals (Guan et al., 1999; Sharma et al., 2007; Gao et al., 2009 and Wang et al., 2009) it might be having adverse effects on pigmentation in fishes on chronic exposure. Keeping these facts into consideration the study was designed to inspect the effect of sub-lethal concentrations of Sodium fluoride on skin pigmentation of *Poecilia reticulata* Peters paralleled with adverse effects on fish behaviour.

MATERIALS AND METHODS:

The test fish, *Poecilia reticulata* Peters measuring 3.5±0.1cm & average weight of 0.52±0.002g were acclimated in the laboratory for two weeks and fed with standard pellet food. The physico-chemical parameters like temperature, pH, DO, Free CO_2, Total hardness, Alkalinity and Acidity of aged tap water were analysed using standard methods as given in APHA (2005). Twenty-five acclimated healthy fish were exposed to three sub-lethal concentrations of Sodium fluoride (NaF) viz. lowest (5.75 ppm), lower intermediate (7.18 ppm) and higher intermediate (9.58 ppm). The doses were selected on the basis of 96 hrs. LC_{50} value being 115 ppm. A duplicate set of this experiment was simultaneously run for confirmation of the results. Control set with same number of test fish but without any toxicant was also run simultaneously. The tests were carried out in glass aquaria measuring 60x30x30cm³ dimensions. The amount of water in each

tank was 2.0 L/g body weight of the test fish. Entire water from each tank was replaced every alternate day to avoid any accumulation of metabolic wastes and to keep the levels of toxicant in the respective tanks constant. At the end of chronic toxicity test period of 15, 30 and 45 days respectively, the surviving fish from control & treatment tanks of three sub-lethal concentrations viz. 5.75 ppm, 7.18 ppm and 9.58 ppm were sacrificed by decapitation, samples of skin (5x5mm^2) were carefully removed from the dorsal body surface of the fish, quickly rinsed in fish saline (0.7% Sodium chloride solution) and immediately fixed in neutral formalin fixative to prevent autolysis and preserve the shape, structure and chemical constituents of the skin tissue. Subsequently excess fixative was removed by washing with 70% alcohol and dehydrated using alcohol grades, cleared in xylene and mounted in Diether Plasticizer Xylene. The number chromatophores were counted and classified using compound microscope. The pooled data (n=6) per concentration and control was further subjected to statistical evaluation using student's t-test. Simultaneously adverse effects on the behaviour of fish was also studied.

RESULTS AND DISCUSSIONS:

The physico-chemical characteristics like Temperature, pH, DO, Free CO_2, Total hardness, Alkalinity and Acidity of aged tap water used in the bioassay study is as presented in Table 1.

Table 1 Physico-chemical Characteristics Of Test Water Used In Bioassay Study

Hydrological Parameters	Range
Temperature (ºC)	29-30
pH	7.2-7.6
Dissolved Oxygen (mg/L)	5.5-6.5
Free CO_2 (mg/L)	Nil
Total Hardness (mg/L as $CaCO_3$)	35-40
Alkalinity (mg/L)	50-55
Acidity (mg/L)	3-4

Behavioral changes exhibited by Guppy, *Poecilia reticulata* Peters:

The exposed fish went through a period of violent movement in circle that intensified with period of exposure and also concentration of the toxicant. Aimless drifting with loss of equilibrium accompanied by copious mucus secretion was also observed to be dose dependent. They preferred the corner of the test chamber with frequent surfacing behavior probably to avoid stress on toxicant exposure. Whereas fishes from control tank preferred to confine themselves to the bottom of the aquarium, swam horizontally and slowly as compared to exposed fish that swam in a slanting manner from lower to upper level. Reduction in food intake and mate chasing behavior was also noted in toxicant exposed fishes when compared to control group of fishes. Such type of behavior can be regarded as the avoidance or adaptive behavior of fish to the Sodium fluoride amplified gulping and increased opercular activity. The behavioral changes in feeding, swimming movement, body orientation, opercular activity, gulping activity, mucus secretion and body coloration were observed in *Clarias batrachus* due to toothpaste, which contained fluoride as one of the content was also reported by Sahu et al. (2014). However Narwaria and Saxsena (2012) too reported similar behavioral response caused by Sodium fluoride induced by Acute Toxicity Bioassay in freshwater fish *Puntius sophore.* The stressful and erratic behavior of fishes in experimental tanks indicates respiratory impairment probably due to Sodium fluoride on gills that also indicates the environmental deterioration.

Structure of Skin and Chromatophores of Guppy, *Poecilia reticulata* Peters:

The skin tissue of Guppy is primarily made up of two principal layers, viz. the outer epidermis of ectodermal origin, non-keratinised consisting of stratified squamous epithelium and inner dermis, which is differentiated into two layers: stratum laxum made up of loose connective tissues interspersed by blood vessels, nerves and sense organs, while the stratum compactum made up of dense connective tissue having bundles of collagen fibres and mesenchyme cells. The epidermis is very thin, usually of 6 to 8 cells thick made up of epithelial cells, lined one above the other. These epithelial cells are continuously shed and replaced. In between the epithelial cells, are the slime cells/unicellular mucous glands with a network of very fine capillaries. These slime cells produce mucoid secretions that form the slime coat. In Guppy, *Poecilia reticulata* Peters vibrant colours, come from the colour cells called chromatophores embedded in the skin above and below the scales. The skin showed abundance of chromatophores especially, melanophores, leucophores and Irridophores (Fig. 1 and 3).

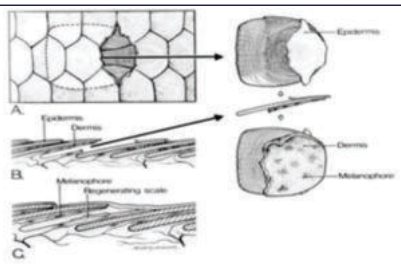

Fig. 1 Structure of Skin and Scale of Guppy (Courtesy: John Gratzek and Janice Matthews).

Melanophores were characterized by cytoplasmic extensions projecting out from the middle of the cell and entwined with the collagen fibres of the connective tissue (Fig. 3).

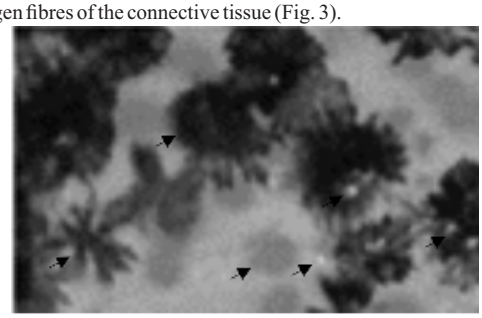

Fig. 3 Structure of skin of *Poecilia reticulata* Peters from control tank (Arrows indicates types of Melanophores (dark) and Irridophores (white) 100x

These cells were observed both in pigment granule dispersion state as well as in pigment granule aggregation state. Location of the pigments within the melanophore characterized the shape of the cell. In dispersion state, the pigment granules were scattered forming dendritic processes. While during aggregation state, all the pigment granules were concentrated in the centre of the cell giving melanophore an ovoid or spherical silhouette. In the present investigation, based on the pattern of dispersion and/or aggregation of pigments in melanophores; reticulate, stellate and punctuate forms have been detected. The leucophores are larger with extremely dendritic processes; their pigment granules are migratory exhibiting a dull whitish colouration.

Effect of Fluoride on Chromatophores of Guppy, *Poecilia reticulata* Peters:

The chronic exposure of *Poecilia reticulata* Peters to Sodium fluoride pragmatically transformed chromatophores morphology i.e. shape, size and also structure as well as dispersion of melanin pigment. There was desquamation of skin epithelium and appearance of haematoma on skin surface due to exposure to Sodium fluoride. Between 15-30 days of exposure period, in lowest (5.75 ppm) and lower intermediate (7.18 ppm) concentration groups recorded increase in number but decrease in size of the chromatophores. Most of the chromatophores were transformed to stellate-shaped in comparison to reticulate-shaped chromatophore in the skin tissue of fish from of control group (Table 2; Fig. 2 and 4).

Table 2: Effect of Sodium fluoride on chromatophores of *Poecilia reticulata* Peters

Concentration of NaF (ppm)	Nature & No. of Chromatophores	Exposure Period (in days)			
		Initial	**15**	**30**	**45**
Control	**Reticulate**	24.44±1.07	29.85±2.01	34.21±0.92	39.32±2.02
	Stellate	21.03±1.76	16.89±0.11	14.24±1.16	11.05±1.04
	Punctate	3.67±1.87	3.99±2.01	4.89±1.26	7.13±0.52
5.75	**Reticulate**	21.12±1.69	26.34±0.57*	31.52±1.94**	34.16±1.86*
	Stellate	22.67±2.03	28.58±0.96*	35.91±1.71*	31.56±1.09**
	Punctate	3.52±1.67	4.98±2.08	7.89±1.86**	9.36±1.32*
7.18	**Reticulate**	22.74±0.89	24.38±2.01*	29.01±1.07*	32.87±2.09*
	Stellate	42.12±2.08	37.54±1.15*	34.69±0.61**	30.15±0.17**
	Punctate	5.48±2.17	8.13±0.73**	10.57±1.31*	11.42±0.39**
9.58	**Reticulate**	25.04±1.08	21.26±2.08*	26.92±2.01*	23.56±1.85**
	Stellate	21.74±0.94	33.78±1.06**	29.17±0.85**	28.92±2.37*
	Punctate	4.24±2.02	8.37±2.11*	12.94±1.62**	13.16±1.93*

Values are Mean ± Std. Error; (N= 6); *p≤0.01; **p≤0.05

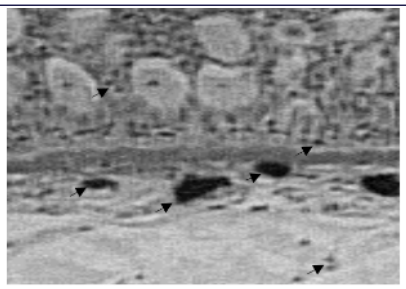

Fig. 2 Structure of skin of *Poecilia reticulata* Peters treated with 7.18 ppm of NaF (Arrows indicates types of Chromatophores) 40x

In higher intermediate (9.58 ppm) concentration of Sodium fluoride after the same exposure span there was diminution in the number of chromatophores. Chromatophores were characterized by reduction in dendritic processes when compared with the control group of fishes with aggregation of melanin towards the centre of the cells. There was intensification in the frequency of breakage of chromatophores as well as dendritic processes both with increasing concentration and period of exposure to Sodium fluoride (Fig. 2). By the end of 45 days of exposure period in lower concentrations there was a slight increase in the number of chromatophore but there was considerable decrease in size. The dendritic processes were almost completely lost or considerably broken or found highly reduced leaving few smaller branches in most of the chromatophores (Fig. 4).

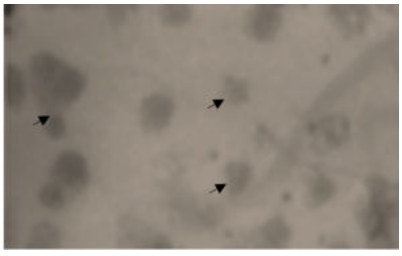

Fig. 4 Structure of skin of *Poecilia reticulata* Peters treated with 9.58 ppm of NaF (Arrows indicates types of Melanophores) 100x

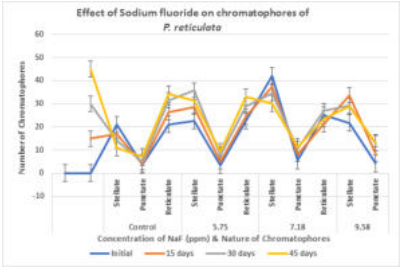

Fig. 5 Effect of Sodium fluoride on chromatophores of *Poecilia reticulata* Peters

Some punctate type chromatophores were also seen in comparison of reticulate type observed in the control group of fishes. Dark coloured fine granules were found liberated within the matrix after breakage of dendritic processes. Most of the chromatophores observed were of deformed shapes with complete loss of the cellular integrity increasing the distance between adjacent chromatophores causing reduction in attractive colouration of the Guppy fish further affecting their reproductive potential, courtship behaviour and mate chasing ability than observed in control group of fishes. The colour change and alterations in morphology of chromatophores observed in the present investigation after fluoride exposure might be due to disruption in endocrine and/or hormonal function as well as due to direct cytotoxic effects caused by intoxication of fluoride.

Chromatophores are accountable for colour changes in fishes at the time of protection, courtship, mating and reproduction (Fujii, 2000). Integumentary melanophores respond instantaneously to contaminants present in the surrounding environment (Singh and Dutta Munshi, 1992). The study on effect of fluoride on colouration in *Herteroneustes fossilis* and *Channa punctatus* respectively found that persistent exposure of Sodium fluoride causes the altered shape, size, dispersion quality of chromatophores in the skin of fish is also apparent in present investigations. Tripathi et al. (2005) and Bajpai and Tripathi (2012) reported that chromatophore numbers increased while their size was reduced and shape of chromatophores become stellate in comparison to reticulate chromatophore of control as also observed in present study. According to Sugimato (2002) melanophores differentiate and die by aptoptosis under the influence of factors that regulate motile responses, which are probably utilizing common intracellular signaling pathways used in part to regulate changes in environment. The mechanism of apoptosis encompasses loss of cell activity, cell fragmentation and phagocytosis of the fragments (Sugimato et al. 2000).

CONCLUSION:

From the present investigation it can be concluded that the behavioral changes observed during the experiment are mainly due to fluoride induces stress that also caused reduction in chromatophore integrity disrupting mating behavior of the fish, thus reducing their reproductive potential under environmental dilapidation.

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