IMIDACLOPRID INDUCED BEHAVIOURAL, THYROID FUNCTIONS AND HEPATIC CHANGES IN EXPOSED AFRICAN CATFISH

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ABSTRACT

Imidacloprid, a systemic neonicotinoid insecticide widely used across the world, has been discovered in numerous freshwater bodies. There are vast information available on the effects of imidacloprid on freshwater fish. This study further assessed the effects of acute doses (0, 10, 30, 50 and 70 mg/L) of imidacloprid on behaviour, swimming, hepatic and thyroid parameters of African catfish (Clarias gariepinus) after 96 hours exposure. Imidacloprid caused significant behavioural alterations (gulping, abnormal surface distribution, under-reactivity, hypoactivity, reduced opercula activity and loss of buoyancy) in the fish. Reduction in swimming speed and distance travelled was observed in fish exposed to imidacloprid. The levels of amylase significantly increased, while alterations were also recorded in the levels of hepatic (ALT, AST, total and conjugate bilirubin and lipase) and thyroid (T3, T4 and TSH) biomarkers. The findings showed that imidacloprid has the potential to harm aquatic species, justifying restrictions on the use of imidacloprid-based pesticides especially near aquatic habitats.

Keywords: Imidacloprid, Behavioural alterations, Hepatic, Thyroid hormone, Catfish

INTRODUCTION

Excessive use of pesticides by the vast population of the developing nations has increased the concentrations of pesticides found in the aquatic ecosystems. Neonicotinoids are a class of emerging contaminants ubiquitously found in soil and water bodies across the globe and causing deleterious effects on humans and non-target organisms like fish in aquatic ecosystems. Imidacloprid is presently one of the major products in many pest control programs (Jeschke *et al.,* 2011; Goulson, 2013). It is used worldwide to control mainly sucking insects on crops and parasites of dogs and cats (Tišler *et al.,* 2009). Although its intended use is not for water, it get to water bodies by run-off or spray drift after application.

Several reports have been made on the presence of imidacloprid in water bodies at various concentrations (van Dijk, 2010; Starner and Goh, 2012; Hrybova *et al.,* 2019). Starner and Goh (2012) reported concentrations of imidacloprid in the range of 1.38 – 3.29 µg/L in the surface waters of three agricultural regions of California. Prior to this, report by van Dijk (2010) showed that higher imidacloprid concentrations (200 µg/L) were detected in the waters of Netherlands. A maximum of 15 µg/L imidacloprid concentration was reported by Kreuger *et al.* (2010) in surface water from streams and rivers running alongside outdoor vegetable crops and greenhouses at different locations in Sweden.

Several studies have been conducted to determine the impacts of imidacloprid on non-

target aquatic organisms (Gauthier *et al.,* 2018; Vignet *et al.,* 2019; Adegun *et al.,* 2020). Imidacloprid has been found to cause reduced viability and hatching success in fish, suggesting that it is more toxic during the early stages of development in fish even at very low concentrations (Adegun *et al.,* 2020). Fish are widely used in environmental pollution studies as indicators and to assess physiological changes and the health of the aquatic ecosystem (Erhunmwunse *et al.*, 2021). This study aims to assess the impact of imidacloprid on the survival, behavioural, swimming, hepatic and thyroid functions of *C. gariepinus*.

MATERIALS AND METHODS

Catfish: Sixty fingerlings of *C. gariepinus* with a mean weight of 0.57 ± 0.08 g and a mean total length 4.41 ± 0.03 cm were used in this study. Fish were purchased from Farm Project, Faculty of Agriculture, University of Benin, Nigeria and transported to the laboratory for Ecotoxicology and Environmental Forensics at the University of Benin, Nigeria. The fish were acclimatized for two weeks prior the commencement of the experiment and kept at 26.40 ± 0.09 °C with 12: 12 hour light-dark photoperiod. The fish were fed with a pelleted commercial diet (Durante Fish Feed, Durante Fish Industries Limited, having 45% crude protein and 3000 Kcal/Kg metabolisable energy) to apparent satiation once daily.

Ethics: All experimental procedures were carried out in conformity with the relevant norms and standards established by the Research Ethics Committee of the College of Medicine of the University of Benin, approval number (CMS/REC/2020/34).

Imidacloprid: Imidacloprid (Jubaili Agrotec Limited, Nigeria) was the toxicant used in this study. The toxicological endpoint of this study was mortality. Four concentrations of imidacloprid (0, 5, 40 and 80 mg/L) were prepared for the range finding test. Nine fingerlings were placed in each concentration after being starved for 24 hours. After 24 hours of exposure, mortality was only recorded in fish exposed to 80 mg/L (22.22%). Mortality occurred when the fish failed to respond to gentle prodding. The dead fish were removed immediately. The median lethal concentration (LC_{50}) was calculated using Finney's Probit analysis spreadsheet calculator (Mekapogu, 2016) based on Finney (1947).

Experimental Design: The experimental design used was a completely randomized design (CRD) of five treatments replicated thrice with each replicate having six African catfish fingerlings. Fifteen glass aquaria (60 L each), with the top cover with nets to prevent escape of the catfish were used for the study. Fish were distributed into the five groups and exposed for 96 hours to 0, 10, 30, 50, 70 mg/L concentrations of imidacloprid. The doses were selected based on the range finding test in a static water system. Fish feeding was halted 24 hours prior to the start of the experiment to reduce the possibility of imidacloprid adsorption.

Culture Conditions: Dechlorinated water was used for the experiment with constant aeration and 80% of the water changed daily. Initial water physicochemical parameters were measured were; temperature (26.40 ± 0.04 °C), dissolved oxygen (5.8 ± 0.82 mg/L), salinity (0.13 ± 0.05 mg/L) and pH (8.19 ± 0.01).

Gross Observation and Behavioural Alterations: During the experimental period, fish were inspected multiple times daily to record behavioural changes and clinical diagnostic signs through direct observation. The physical appearance of fish in each aquarium was monitored, and changes such as malformations and changes in body color were recorded in comparison with the control treatments. Behavioural changes such as reactivity to stimuli, surface distribution, gulping, opercula activity and buoyancy were monitored.

Swimming Speed/Distance Covered: The methods used by Erhunmwunse *et al.* (2021) were used to determine the swimming speed of the fingerlings. Video recordings with a digital camera (Nikon D3100) were first used to ascertain the swimming patterns and pace of

the fingerlings at 96 hpf. The video files were processed by the Kinovea (0.9.5) software to produce the video tracks. The fish's swimming speed per minute was determined by dividing the distance travelled by the swimming duration (60 seconds).

Sampling: At the conclusion of the experiment, three catfish were randomly selected from each replicate and anesthetized with 100 mg/L of benzocaine solution. Biochemical analysis was done to evaluate tissue damage. The whole fish tissue (0.1 g) were mixed in mortar and pestle with ice-cold distilled water (0.9 mL) to make a homogeneous mixture, then centrifuged at 3000 x g for 20 minutes at 4 °C to obtain the supernatant which was then used for further analysis (Das *et al.*, 2012).

Hepatic and Thyroid Functions Assay: Alanine aminotransferase (ALT) and Aspartate aminotransferase (AST) were determined using the colorimetric method as described by Reitman and Frankel (1957). The ALT was measured by monitoring the concentration of pyruvate hydrazone formed with 2,4-dinitrophenyl-hydrazine, while AST was measured by monitoring the concentration of oxaloacetate hydrazone formed with 2,4-dinitrophenyl-hydrazine.

Bilirubin was determined using the colorimetric method as described by the modified Jendrassik and Grof's method (Doumas *et al.*, 1985). Azubilirubin, a purple-colored complex whose absorbance is proportional to bilirubin content, is created when bilirubin combines with diazotized sulfanilic acid in acidic medium. To further account for the unconjugated or indirect bilirubin, the diazotization is conducted briefly for 30 minutes in the presence of an activator (caffeine reagent).

Amylase was determined using the procedure described by Bernfeld (1955). Maltose was used as a standard for the measurement of absorbance at 600 nm, and the amount of amylase that produced one mmol of maltose ml^{-1} min^{-1} was defined as the activity unit of the enzyme. Lipase activity was determined according to Bier (1955) based on evaluation of the enzymatic hydrolysis of diacylglycerols, monoacylglycerols and triacylglycerols to free fatty acids in emulsion of olive oil. The hydrolysis of 1.0 microequivalent of fatty acids from triacylglycerols in 1 hour at pH 7.7 and 37 °C was used to define one unit of lipase activity.

The determination of serum level of T3 and T4 was done by using ELISA kit according to manufacturer's instruction (Calibiotech, Inc., California, USA). The values of control and experimental samples were read within 10 minutes using microplate reader (BIO-RAD) with a 450 nm filter. TSH levels were analyzed using ELISA test kits by microplate immune enzymometric assay. The samples and Biotin labelled anti-TSH-HRP conjugate were added to the wells coated with streptavidin TSH in the serum bound to anti-TSH and forming a sandwich with streptavidin coated wells. Unbound proteins were washed off by wash buffer. Upon the addition of the substrate, the intensity of color is proportional to the concentration of TSH in the samples. The absorbance was measured at 450 nm against a blank.

Statistical Analysis: Data were statistically analysis using one way analysis of variance (ANOVA) followed by post hoc Dunnett's multiple comparison test. The level of significant difference was set at $p<0.05$. GraphPad Prism (Version 5.01) was used to analyze the data.

RESULTS

Mortality: The mortality rate was concentration and time dependent, with percentage mortality peaking at 61.11% after 96 hours of exposure to imidacloprid. Mortality was observed at concentrations ≥ 50 mg/L. (Table 1). The 96 hours LC_{50} of imidacloprid was 64.88 mg/L (95% confidence intervals: 55.29 – 76.13 mg/L).

Behavioural Responses: The behavioural observations of the fish were carried out at 24 and 48 hours. The fish in the control behaved naturally, being active with well-coordinated movements and were alert to the slightest disturbance.

Table 1: Cumulative mortality of *Clarias gariepinus* fingerlings exposed to various concentrations of imidacloprid for 96 hours

Imidacloprid concentration (mg/L)	Number of exposed fish	Cumulative mortality of fish per time interval (hours)				Mortality %
		24	48	72	96	
0	18	0	0	0	0	0
10	18	0	0	0	0	0
30	18	0	0	0	0	0
50	18	1	2	3	3	17
70	18	2	3	9	11	61

For the fish exposed to imidacloprid, however, there were observed deviations in their behaviour from those in the control with the impact of imidacloprid on this deviation increasing with both increasing concentration and time of exposure. The behavioural abnormalities which were observed were air gulping and abnormal surface distribution (Figures 1 and 2) and under-reactivity and hypoactivity to stimuli (Figures 3 and 4).

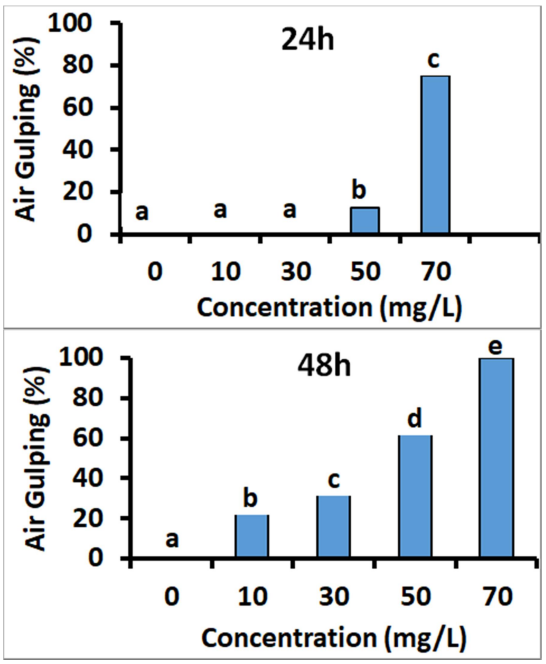

Figure 1: Percentage of behavioural response of *Clarias gariepinus* to varied concentrations of imidacloprid through air gulping after 24 and 48 hours of exposure. ***Key:*** *Concentrations on the same graph with different letter superscript are significantly different (p<0.05)*

The fish opercula activity also decreased with increasing concentration of imidacloprid and time of exposure. Loss of buoyancy was also observed just before the death of a fish due to exposure to imidacloprid.

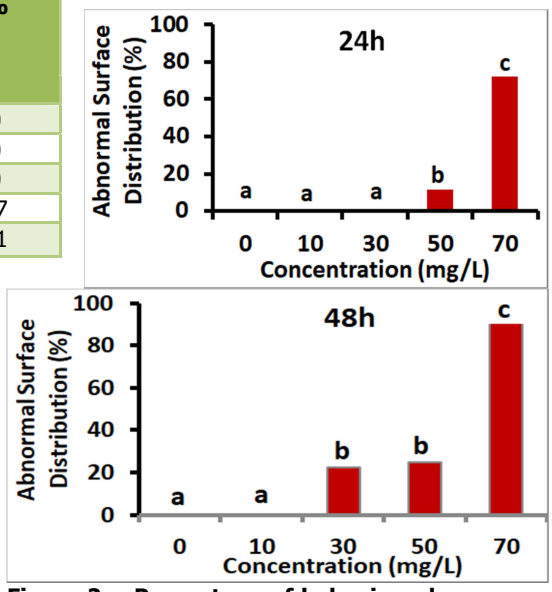

Figure 2: Percentage of behavioural response of *Clarias gariepinus* to varied concentrations of imidacloprid through abnormal surface distribution after 24 and 48 hours of exposure. ***Key:*** *Concentrations on the same graph with different letter superscript are significantly different (p<0.05)*

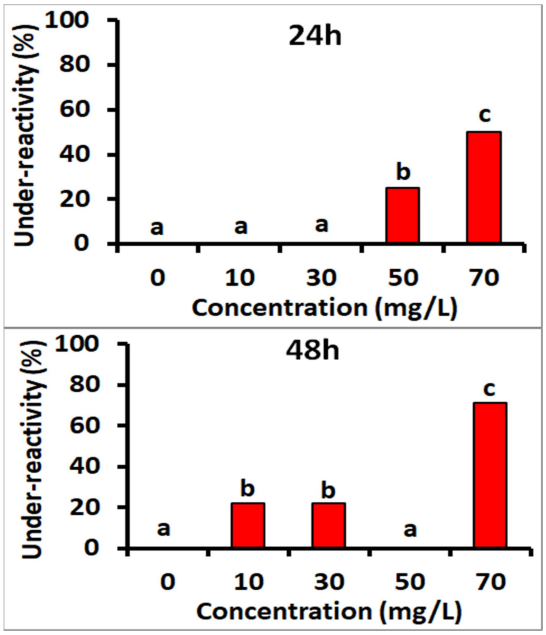

Figure 3: Percentage of behavioural response of *Clarias gariepinus* to varied concentrations of imidacloprid through under-reactivity after 24 and 48 hours of exposure. *Key:* *Concentrations on the same graph with different letter superscript are significantly different (p<0.05)*

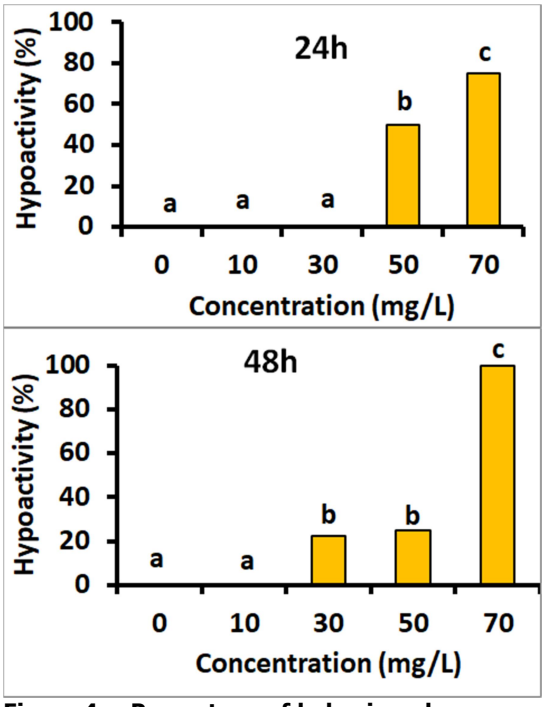

Figure 4: Percentage of behavioural response of *Clarias gariepinus* to varied concentrations of imidacloprid through hypoactivity after 24 and 48 hours of exposure. ***Key:*** *Concentrations on the same graph with different letter superscript are significantly different (p<0.05)*

Swimming Speed/Distance Covered: *C. gariepinus* fingerlings exposed to imidacloprid at 96 hpf demonstrated a significant reduction in swimming speed and distance travelled (p<0.05). The swimming speed of the fingerlings in the control group was significantly faster than that of the exposed groups, which revealed a significant decline (p<0.05) in a concentration-dependent manner (Figure 5).

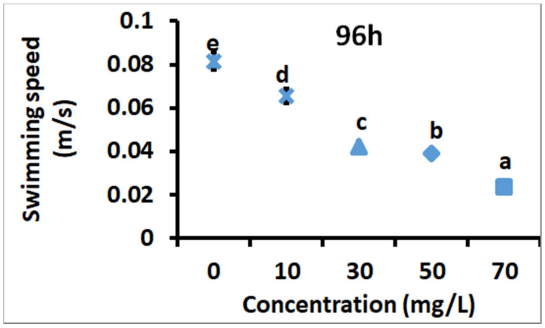

Figure 5: Average swimming speed of *Clarias gariepinus* fingerlings exposed to imidacloprid at 96hpf. ***Key:*** *Column graph indicate mean ± SE (n=6). Means on the same graph with different letter superscript are significantly different (p<0.05)*

At 96 hpf, the swimming speed of the fingerlings exposed to imidacloprid at concentrations of 30 mg/L (0.040 ± 0.002 m/s), 50 mg/L (0.036 ± 0.003 m/s), and 70 mg/L (0.025 ± 0.001 m/s) was significantly (p<0.05) lower than that of the control group (0.078 ± 0.003 m/s). The greatest reduction in distance travelled was seen in larvae subjected to 70 mg/L of imidacloprid, which was about three times less than the distance travelled by the control fingerlings (Figure 6).

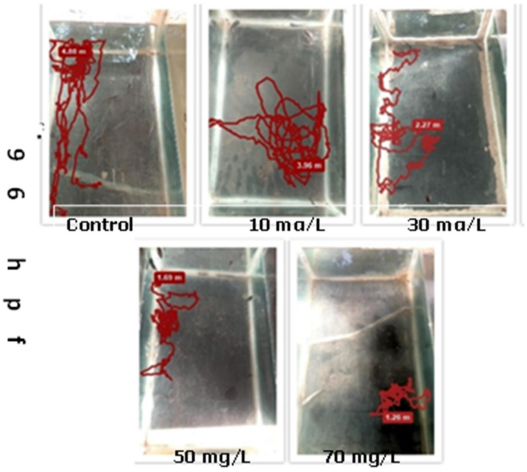

Figure 6: *Clarias gariepinus* fingerlings video tracks showing the swimming distance travelled in different concentrations of imidacloprid at 96 hpf for 60 s. ***Key:*** *The red rectangle indicates the endpoint of each video track while the red lines show the movement tracks (n = 6)*

Hepatic Functions: The serum levels of the hepatic variables (ALT, AST, total and conjugate bilirubin, amylase and lipase) of *C. gariepinus* exposed to different concentrations of imidacloprid for 96 hours showed a significant increase ($F = 6.24$, $p = 0.04$) in amylase levels (Table 2). None of the changes in the levels of ALT, AST, total bilirubin, conjugate bilirubin and lipase in all concentrations were significant (p>0.05) compared to the control.

Thyroid Functions: The levels of thyroid hormones (T3, T4 and TSH) of *C. gariepinus* exposed to different concentrations of imidacloprid for 96 hours showed a decrease in T3 and T4 levels, and an increase in TSH levels, in *C. gariepinus* (Table 3). These changes were not significantly different (p<0.05) from the control in all concentrations of imidacloprid.

Table 2: Hepatic variables of *Clarias gariepinus* exposed to different concentrations of imidacloprid for 96 hours

Imidacloprid (mg/L)	ALT (U/L)	AST (U/L)	Total bilirubin (µmol/L)	Conjugate bilirubin (µmol/L)	Amylase (U/L)	Lipase (U/L)
0	2.10 ± 0.20	193.70 ± 2.60	0.28 ± 0.07	0.11 ± 0.07	6.36 ± 1.06^{a}	0.05 ± 0.00
10	6.00 ± 1.40	189.80 ± 13.85	0.20 ± 0.08	0.07 ± 0.02	10.07 ± 0.53^{b}	0.64 ± 0.00
30	5.60 ± 0.50	162.60 ± 34.25	0.31 ± 0.03	0.06 ± 0.03	26.50 ± 5.30^{d}	0.41 ± 0.12
50	4.95 ± 0.15	209.70 ± 1.85	0.20 ± 0.03	0.12 ± 0.02	15.37 ± 3.71^{bc}	0.35 ± 0.23
70	9.95 ± 4.15	206.30 ± 3.70	0.17 ± 0.01	0.12 ± 0.04	19.61 ± 2.65^{c}	0.58 ± 0.12

Means on the same column with different letter superscript are significantly different ($p<0.05$)

Table 3: Thyroid variables of *Clarias gariepinus* exposed to different concentrations of imidacloprid for 96 hours

Imidacloprid (mg/L)	T3 (ng/mL)	T4 (µg/dL)	TSH (µlm/mL)
0	0.74 ± 0.17	3.40 ± 0.30	0.75 ± 0.15
10	0.50 ± 0.00	3.00 ± 0.00	1.30 ± 0.10
30	0.70 ± 0.10	3.10 ± 0.00	1.10 ± 0.10
50	0.58 ± 0.02	3.55 ± 0.45	1.45 ± 0.15
70	0.55 ± 0.05	3.00 ± 0.00	1.30 ± 0.30

DISCUSSION

Pesticides can have a negative effect on the environment and non-target terrestrial and aquatic animals. They can interrupt the normal physiological processes and alter the progress of animals' reproduction and life cycle. In this study, we demonstrate how imidacloprid, a widely used insecticide in agriculture, can affect the mortality, behaviour, hepatic functions and thyroid functions of *C. gariepinus*. These findings are interesting as they upgrade our knowledge of the effect and processes by which imidacloprid acts on fish.

Behaviour is a visible response of an organism to a stimulus on the whole-organism organization level (Desai and Parikh, 2014). However, it is highly integrative as it is based on biochemical reactions ad exerts impacts on the population and biocoenosis levels (Dell'Omo, 2002). *C. gariepinus* showed behavioural changes on exposure to imidacloprid. Typically, in most fishes, acute toxicity is characterized by behavioural changes such as excessive gulping of air, intermittent swarming, erratic swimming, loss of movement, erratic swimming, jerky movement, excessive secretion of mucus and altered body pigmentation (Inyang *et al.*, 2017; Oyoroko and Ogamba, 2017). According to Lebedeva *et al.* (1998), external mucus is a result of metabolic processes that occur in the fish organs, which may also be a criterion of the physiological status of the fish establishing the special effects that various factors like toxicant and the environment produce on it. Mucus accumulation on gills impedes respiratory activity which hinders the gill surface from carrying out active gaseous exchange and leads to the death of the fish (Evans *et al.*, 2005). Srivastava *et al.* (2010) reported that the accumulation of mucus on the gills of *Heteropneustes fossilis* hindered gill functions causing an internally toxic environment from the build-up of nitrogenous wastes in the body resulting in death. The behavioural changes shown by fish may have been a response to aquatic hypoxia condition (Kind *et al.,* 2002) caused by imidacloprid. When it is impossible to escape from hypoxic stress, physiological changes may be evoked to make up for the low oxygen supply (Val *et al.,* 1998). The gulping of air may help to avoid contact with toxic medium (Patil and David, 2008). Surfacing phenomenon i.e., significant preference of upper layers in the exposed group might be a demand for higher

oxygen levels during the exposure period (Schmidt *et al*., 2005).

In aquatic vertebrates and invertebrates, swimming behaviour is a complex endpoint used to evaluate toxicity (Bownik *et al*., 2017). Our study indicated that although *C. gariepinus* could survive at imidacloprid concentrations up to 30 mg/L, the fingerlings responded after 96 hours with inhibition of swimming distance and speed. This could be due to the association between imidacloprid and nicotine receptors resulting in the inhibition of neuronal transmission and reduction of swimming speed and distance, which inhibits motility (Bownik *et al*., 2017). Our findings also showed that *C. gariepinus* appears to use nicotinic receptor signalling as a key pathway for neuromotor action. These findings are similar to those of Erhunmwunse *et al*. (2023) who found *C. gariepinus* larvae after 48 hours exposure to imidacloprid exhibited reduced swimming speed and distance. Similarly, imidacloprid was reported to reduce swimming speed and distance travelled in adult *Danio rerio* (Guerra *et al*., 2021) and *Labeo rohita* fingerlings (Qadir *et al.,* 2014) after 96 hours exposure. The reduced swimming speed and distance travelled by catfish fingerlings is a behavioural indicator of imidacloprid's toxic impacts.

In this study, ALT levels of *C. gariepinus* increased on exposure to acute concentrations of imidacloprid, peaking at the highest imidacloprid concentration, 70 mg/L. Alanine transaminase and aspartate transaminase are actively involved in the breakdown of proteins and amino acids (Vroon and Israili, 1990). The enzymes are leaked out of the cells if any tissue is damaged, to neutralize the toxic substances (Giannini *et al*., 2005). Increased levels of these enzymes may be associated with hepatic injury in fish (Rastiannasab *et al.,* 2016). The results obtained demonstrated that imidacloprid can inhibit the metabolism of protein and amino acids in the liver.

Bilirubin is produced by the breakdown of heme, a component derived from the haemoglobin of red blood cells or other haemoproteins, such as myoglobin, cytochromes and catalase. Heme oxygenase (HO), a rate-limiting enzyme in heme catabolism, cleaves heme to form biliverdin, which is subsequently converted by biliverdin reductase into bilirubin (Ayer *et al.,* 2016; Tsai and Tarng, 2018). The change in bilirubin levels suggests liver cell damage (Singh *et al*., 2022). The reduction in values of total bilirubin could be attributed to the liver being unable to convert bilirubin into bile and urobilin (Singh *et al*., 2022).

Amylase activity increased significantly in the exposed fish. Amylase is secreted by the exocrine region of the pancreas. It catalyzes the conversion of starch to maltose. The increased activity may be a result of pancreatitis or damage of the amylase secretary cells (Yousafzai and Shakoori, 2011). An increase in amylase and other liver enzymes indicate acute liver damage (hepatitis) in the fish (Yousafzai and Shakoori, 2011). It also indicates an abnormality of the digestive process (Sanna *et al.,* 2021). The increase in amylase could have been due to the fish adjusting themselves by accelerating the enzyme to improve the metabolism of carbohydrates in order to provide extra energy for the cells (Inyang *et al.,* 2016).

The levels of lipase increased on exposure to imidacloprid in this study. This increase indicates serious tissue damage due to the increased production of the enzyme in the tissues (Samanta *et al.,* 2016). During stress conditions, food intake is less, therefore, the activity of digestive enzymes such as lipase may be enhanced to break down lipid molecules to overcome this situation and fulfill the extra energy requirement (Samanta *et al.,* 2016).

Many fishes respond to stressors by showing endocrine shifts (Sanna *et al.,* 2021). Thyroid hormones are important in the development and growth of fish, particularly during their early life stages, thus thyroid disruption by exposure to environmental toxicants could inhibit the growth of fish larvae and juveniles and reproduction in adults (Nugegoda and Kibria, 2017). Thyroid hormones are unique in affecting almost every tissue of the body through life (Shahid *et al*., 2022). Imidacloprid may cause a decrease in basal metabolism due to the decreased levels of T3 and T4 (Shah *et al.,* 2014), causing abnormalities

in growth, development, reproduction, and behaviour (Pandey and Mohanty, 2015). Elevations in serum TSH levels with increasing dose exposure may be due to negative feedback of reduced amount of circulating T3 and T4 at the peripheral level to hypothalamo-pituitary-thyroid (HPT) axis (Dey and Saha, 2014). In fish, thyroid hormones play a crucial role in the control of growth, development, metamorphosis, reproduction and behaviour (Rafieepour *et al.,* 2019).

Conclusion: This study clearly showed that imidacloprid has the potential to disrupt hepatic and thyroid functions in fingerling fish. Imidacloprid exposure also induced a series of negative impacts on fingerling catfish behaviour, swimming ability and mortality. These findings suggest that catfish fingerlings exposure to imidacloprid may hinder developmental and growth success from fingerlings into later stages of development. Therefore, it is crucial to regulate the use of imidacloprid based insecticides, especially when they are used close to aquatic habitats.

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