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RESEARCH ARTICLE

Sublethal behavioral and biochemical toxicity of cypermethrin in juvenile *Oreochromis niloticus* in a static bioassay

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Abstract. Sublethal behavioral and biochemical toxicity of cypermethrin in *Oreochromis niloticus* (L.) juveniles was accessed under static conditions at concentrations of 0.5, 1.1, and 2.1 μg L^{-1} for up to eight weeks. The juveniles were highly sensitive to cypermethrin, with a 96-h LC_{50} of 12 μg L^{-1}. Biphasic trends were noted for four avoidance behaviors: loss of equilibrium; erratic swimming; air gulping; opercular ventilation. Dose- and duration-dependent increases occurred in mucus secretion and color change. Mixed trends were noted for mean glutamate pyruvate transaminase, triglycerides, proteins, and cholesterol. These parameters decreased significantly depending on dose and duration in weeks two and six, but they increased in week eight. Mean glucose and glutamic oxaloacetic transaminase increased significantly depending on dose and duration up to week eight. No differences in glucose were noted in the control in week two. The findings of the present study confirmed that cypermethin adversely affected the health of fish even at a sublethal level.

Keywords: behavior, biochemistry, cypermethrin, fish, freshwater, toxicity.

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Introduction

Cypermethrin (CYP) is a commercial synthetic pyrethroid that farmers use widely in Nigeria. It is a systemic and contact pesticide that is effective against a wide range of insect pests particularly Lepidoptera in cereals, citrus, cotton, forestry, fruits, soyabeans, tobacco, tomatoes, vegetables, and other crops (Charles and Hance 1968). In 1988, pyrethroids accounted for 40% of the sales of insecticides for cotton treatment in the world, to which CYP contributed 8% (Allan Woodburn Associates 1995). CYP has been used in the past and especially recently to impregnate mosquito bed nets to prevent malaria and also to control indoor pests. In Africa and in other parts of the world, pyrethroids have proven successful as a malaria control method, as the use of insect-treated nets has been correlated with a 27% reduction in the risk of post-neonatal deaths (Lindsay and Martens 1998). However, the extensive use of pyrethroids, and particularly CYP, endangers aquatic life (Seth and Saxena 2004). CYP is particularly harmful in water to fish, marine invertebrates, and other living species. The acute and chronic neurotoxicity of CYP is mainly mediated by central nervous system (CNS) hyper-excitation by inhibiting sodium channels (Haque and Mondal 2016). According to Manna et al. (2005), CYP is neurotoxic by

modulating the levels of α-aminobutyric acid (GABA), an inhibitory neurotransmitter. CPY also controls chloride, potassium-gated voltage, and calcium channels, modulates glutamate activity–an excitatory neurotransmitter, acetylcholine receptors, and adenosine triphosphates, and induces oxidative stress in neuronal cells leading to DNA damage (Singh et al. 2012). Since CYP-induced oxidative stress leads to dopaminergic neurodegeneration that leads to motor dysfunction, CPY can be considered to be one of the pesticides involved in the pathogenesis of Parkinson's disease (Nasuti et al. 2007, Singh et al. 2012). CYP is easily hydrolyzed by microsomal carboxylesterase into inactive acid and alcohol components in the liver (Houston 1997). The slow rate of detoxification is a major factor in the sensitivity of fish species to pyrethroids.

Drummond et al. (1986) stated that behavioral and morphological changes in fish can be used according to their mode of action as a diagnostic endpoint for screening and differentiating chemicals. After exposing 30-day old fathead minnows, *Pimephales promelas* Raf. to various chemicals, they concluded that selecting abnormal responses is promising for predicting the mode of action of unknown xenobiotics. Abnormal behavior exhibited by fish can be caused by abnormal neurotransmitter levels, and such modifications occur much earlier than mortality (Ogueji et al. 2019). Pesticides are deposited in tissues such as the liver and muscle and contribute to organ failure resulting in mortality (Srivastava and Kaushik 2001). Various enzymes are essential for normal biological function, and interventions in enzyme activity act as primary indicators of contaminant toxicity (Jensen et al. 1991). Ogueji et al. (2018) states that while there is some data available on the impact of pesticides on vertebrates in Nigeria, there is very little data available on the impact of pesticides on fish. In the region where the research was carried out, CYP is used widely for insect control in agriculture, so it can enter fish ponds and natural bodies of water, and this is why toxicity studies are required.

The purpose of this study was to evaluate the toxic effects of sublethal CYP concentrations on serum biochemistry and behavioral and morphological indices of *Oreochromis niloticus* (L.), a common African indigenous tropical freshwater cichlid that is prevalent and in high demand.

Materials and methods

Test fish and experimental pesticide

Nile tilapia, *O. niloticus*, juveniles of mixed sexes and fairly uniform size were obtained from the Kubani Dam, Ahmadu Bello University, Zaria. The fish were transported to the fisheries laboratory in a portable, well-aerated white polythene bag containing water from the dam. They were held in large tanks with a 160 L capacity at 24.5-25.5°C and acclimatized for two weeks in dechlorinated municipal water. Air pumps were used to aerate all the tanks to maintain optimum dissolved oxygen concentrations. During the acclimatization period, the fish were fed commercial 4 mm Coppens fish feed (Afrimash, Lagos, Nigeria) containing 45% protein twice daily (9:00 and 18:00). CYP [cyano-3-phenoxybenzyl- (1R, S, cis, trans)-2, 2-dimethyl-3, (2, 2-dichlorovinyl), cyclopropane1carboxylate] (Comfort Agrochemicals, Zaria, Nigeria) with 32% active ingredient, 4 $\mu g\ L^{-1}$ at 20°C, was dissolved in distilled water to formulate a stock solution. Three sublethal concentrations (0.5, 1.1, and 2.1 $\mu g\ L^{-1}$) of CYP were prepared from the stock solution using distilled water. Sublethal concentrations of CYP were renewed every 48 hours with freshly prepared stock solutions.

Determination of sublethal concentrations

From the results of the static acute bioassay, a fraction of 1/5, 1/10, and 1/20 of the 96-h LC_{50} (12.6 $\mu g\ L^{-1}$) obtained by Yaji et al. (2011) was used to determine the sublethal concentrations (0.5, 1.1, and 2.1 $\mu g\ L^{-1}$) for CYP as recommended by Ogueji (2008). Measurements for the toxicity test were done following the procedure in Sprague (1969).

Experimental design

Four tanks of the same size (30.5 cm × 30.5 cm × 46.25 cm) were used for three replicates of each of the toxicants (12 tanks). Appropriate volumes of the stock solution were dispensed using micro-syringes into 25 L of de-chlorinated municipal water in each of the test tanks, except the control. This was renewed every 12 h, which helped to maintain toxicant strength and the level of dissolved oxygen, and it also minimized ammonia levels during the experiment (Ogueji et al. 2018). The juveniles were exposed to sublethal concentrations of toxicant for eight weeks. The concentrations used for chronic study of the toxicant were 0.5, 1.1, 2.1 μg L^{-1}, and 0.00 μg L^{-1} (control). Each treatment was in triplicate. The fish (25 ± 0.52 g and 8.9 ± 0.65 cm) were assigned randomly at a density of 10 fish per tank. The fish were fed rations of 3% body weight with the commercial 4 mm Coppens fish diet (with 45% crude protein). The treated fish were sampled in weeks two, six, and eight to determine toxic effects. A natural photoperiod of 12 h light :12 h dark was maintained.

Water quality test

Some properties of the test water were monitored and analyzed daily, and remnants of unconsumed feed and excreta were siphoned out. Following the procedure of APHA (1985), the water quality parameters monitored and estimated were temperature, pH, and dissolved oxygen. The means for the parameters were as follows: temperature 28.35 ± 0.12°C; pH 6.01 ± 6.47; oxygen 5.65 ± 0.14 mg L^{-1}.

Behavioral and morphological assays

Observations of behavioral and morphological responses of *O. niloticus* juveniles were conducted at weeks two, six, and eight during the sublethal toxicity tests according to Drummond et al. (1986). In addition to the sublethal doses, controls without toxicant exposure were tracked to provide a guide for the evaluation of any behavioral and phenotypic changes. Responses were registered if they differed from the controls and if they occurred in 10% of the fish. The responses monitored included loss of balance, irregular swimming, excessive mucus secretion, abnormal opercular ventilation, increased air gulping, and altered skin coloration. Each test tank was observed for 5–10 min. Startle responses were tracked with tactile stimulus by gently touching the fish with a plastic applicator stick.

Blood and serum collection

Blood (3 mL) for biochemical examination was drawn according to Bello et al. (2014) in 5 mL non-heparinized tubes that were immediately taken to Ahmadu Bello University Teaching Hospital, Chemical and Pathology Department. Two fish per treatment were selected for blood collection in each sampling period. The fish were not sacrificed, and the blood was drawn easily by immobilizing them. This was done by puncturing the fish with needle at a distance of 3–4 cm from the genital opening that had been wiped with soft sanitary towel to avoid mucus contamination. The needle was inserted at a right angle to the vertebral column of the fish and blood was drawn under gentle aspiration until about 3 mL was obtained. The needle was withdrawn gently and the blood was transferred into an anticoagulant plastic tube. After the blood was drawn, the fish were put in separate tanks to recover and later returned to the test tanks. To obtain the serum, the blood was placed in micro centrifuge tubes, and immediately centrifuged at 1500 rpm for 10 minutes. Serum was then removed by pipetting and determinations were performed immediately of glucose, total protein, glutamic oxalo-acetic transminase (GOT), glutamate pyruvate transaminase (GPT), triglycerides, and cholesterol levels with an automatic biochemical analyzer (Olympus AU 400 biochemical analyzer, Tokyo, Japan) following the manufacturer's instructions.

Statistical analysis

Biochemical data were analyzed using the statistical package IBM SPSS (Version 20). One-way analysis of

Table 1
Effect of cypermethrin on behavioral indices of *O. niloticus* at different exposure durations

Concentrations	Loss of Equilibrium	Abnormal mucus secretion	Erratic swimming	Air gulping	Abnormal opercular ventilation	Change in skin coloration
14 days						
Control	-	-	-	-	-	-
0.5 μg L^{-1}	-	-	-	-	-	-
1.1 μg L^{-1}	-	-	+	+	+	-
2.1 μg L^{-1}	+	+	++	++	++	+
42 days						
Control	-	-	-	-	-	-
0.5 μg L^{-1}	-	-	-	-	-	-
1.1 μg L^{-1}	+	+	++	++	++	+
2.1 μg L^{-1}	++	++	+++	+++	+++	++
56 days						
Control	-	-	-	-	-	-
0.5 μg L^{-1}	-	-	+	+	+	+
1.1 μg L^{-1}	+	++	+	++	++	++
2.1 μg L^{-1}	++	+++	++	++	++	+++

None (0%); + Mild (<10%); ++ Moderate (10–50%); +++ Strong (>50%).

variance was used to determine whether there were any significant differences between the means of the sublethal concentrations at different exposure durations. Results were presented as mean ± SE. Tests for significant differences among treatment groups were done with the Duncan multiple range test, and significance was declared at a 5% level.

Results

Change in fish behavior

During the sublethal exposure period, normal behavioral activities were noted in the control and the group treated for two weeks. Dose and duration-dependent abnormal changes characterized by loss of equilibrium, abnormal mucus secretion, erratic swimming, abnormal opercular ventilation, increased air gulping, and changes in skin coloration were observed in the CYP-treated fish (Table 1). The three behavioral indices of erratic movement, opercular ventilation, and increased air gulping were moderately (10-50%) and strongly (>50%) dependent on duration at the highest sublethal concentration (2.1 μg L^{-1}) in weeks two and six; however, in week eight the responses weakened at the same sublethal concentration (Table 1). Each test tank was observed for 5–10 min.

Serum biochemical response

The effects of CYP exposure on serum biochemical parameters (Fig. 1) at two, six, and eight weeks were significantly ($P < 0.05$) dose-dependent increases in GOT with the highest value of 810.7 ± 5.8 (IU L^{-1}) recorded at week six at 2.1 μg L^{-1}. GPT, alternatively, showed significant ($P < 0.05$) dose-dependent decreases at two and six weeks of exposure. The fish exposed to 0.5 μgL^{-1} exhibited the highest value of GPT at 760 ± 35 (UI L^{-1}) in week six. This trend shifted at eight weeks of exposure when there were dose-dependent increases of GPT that were significant ($P < 0.05$) when compared to the control.

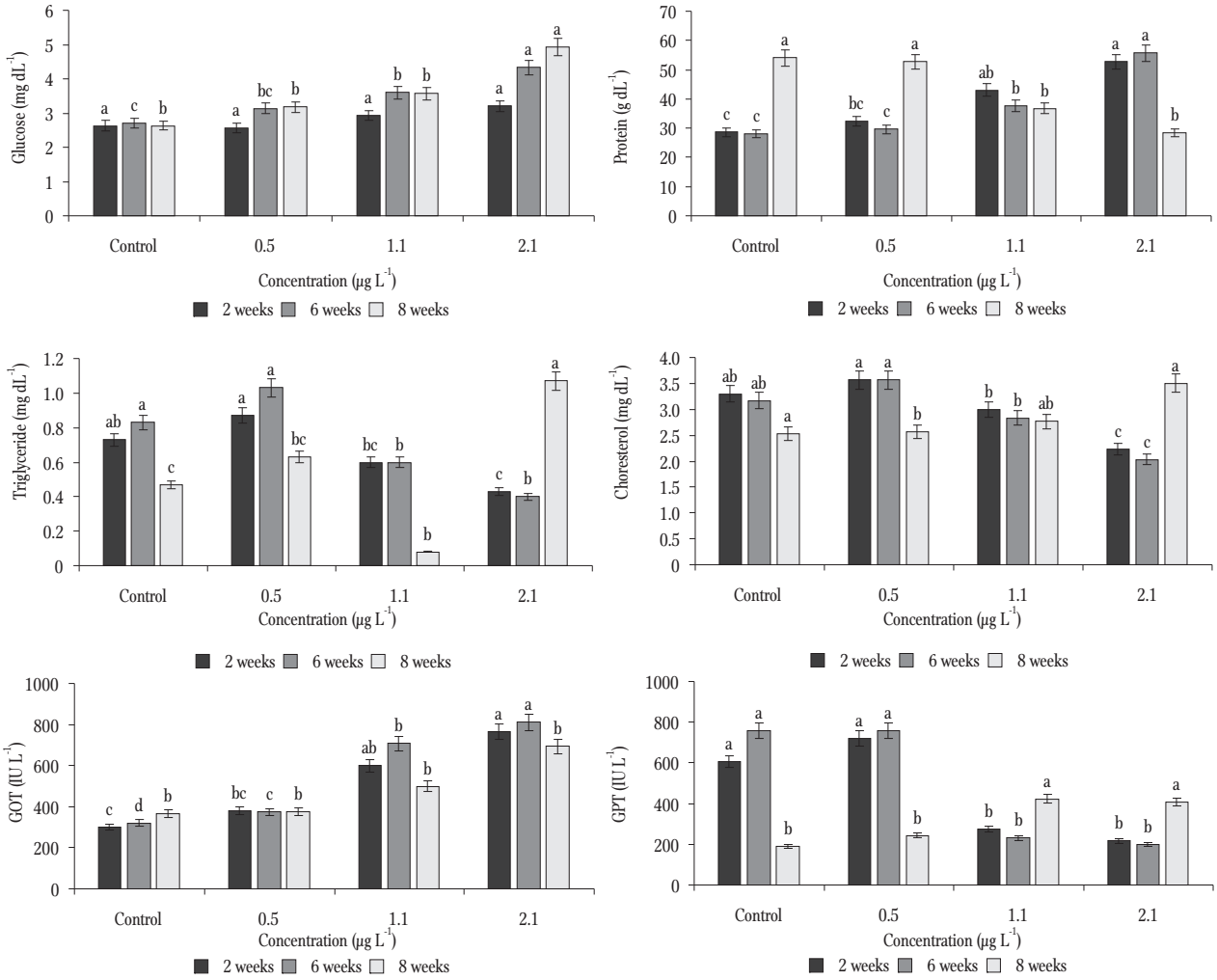

Figure 1. Biochemical responses (glucose, protein, cholesterol, triglyceride, GOT, and GPT) in *O. niloticus* exposed to sublethal concentrations of cypermethrin in a static bioassay for two, six, and eight weeks. Different letters indicate significant differences ($P<0.05$) in mean values of different concentrations and the control. Error bars denote SE.

Dose-dependent decreases in triglyceride and cholesterol were significant ($P < 0.05$) in weeks two and six of exposure. Decreases were recorded for 1.1 and 2.1 µg L^{-1} CYP. However, in week eight of exposure, CYP elicited significant ($P < 0.05$) increases at concentrations of 1.1 and 2.1 µg L^{-1}. Dose-dependent increases in protein compared to the control were significant ($P < 0.05$) in weeks two and six of exposure. However, in week eight of exposure protein decreased significantly ($P < 0.05$) at concentrations of 1.1 and 2.1 µg L^{-1}. Significant ($P < 0.05$) dose-dependent increases of glucose in comparison with the control were noted in weeks six and eight of exposure. The increases in week two were not significant.

Discussion

96-h LC_{50} value of CYP for juvenile *O. niloticus*

A review of the literature suggested that CYP is extremely toxic to *O. niloticus* juveniles at a 96-h LC_{50} value of 12.6 µg L^{-1} (Yaji et al. 2011). Similar findings were published for *O. niloticus* and other fish

species including *O. niloticus* juveniles with a 96-h LC_{50} of 82 μg L^{-1} (Yuniari et al. 2016), adult *O. niloticus* at 5.99 μg L-1 (Sarikaya 2009), juvenile *Clarias gariepinus* (Burchell) at 19 μg L^{-1} (Ogueji 1997), fingerling *Labeo rohita* (Hamilton) at 4.0 μg L-1 (Marigoudar et al. 2009), and juvenile *Heteropneustes fossilis* (Bloch) at 0.25 μg L^{-1} (Singh and Zahra 2017). Factors that might have influenced variations in 96-h LC_{50} values of fish to CYP included fish age, the physical and chemical parameters of the experimental water, fish hardiness, exposure duration/concentrations, pH, dissolved oxygen, formulation of the pesticide tested, biotransformation/excretion, and the heterogeneous pesticide metabolism of individual fish species (Ogueji et al. 2018). David et al. (2004) reported that CYP was extremely toxic to fish at very low concentrations with 96-h LC_{50} in the range of 0.4–2.2 μg L-1. Sublethal exposure of *O. niloticus* to CYP adversely affected the health of fish at both biochemical and behavioral levels, which is in agreement with the acute toxicity threshold reported in the literature cited above.

Behavioral responses to sublethal exposure to CYP

Two basic explanations as to why CYP causes behavioral changes are that it might pass through the fish blood-brain barrier and exert CNS neurotoxicity and also trigger motor deficits (Akhtar et al. 2021). CYP induces neurotoxicity and causes oxidative stress resulting in neuronal cell DNA damage by modulating levels of GABA, chloride, voltage-gated potassium and calcium channels, glutamate (excitatory neurotransmitter) function, acetylcholine receptors, and adenosine triphosphates (Singh et al. 2012). From the outcome in Table 1, CYP induced dose- and period-dependent behavioral changes in *O. niloticus* juveniles that were characterized by loss of balance, irregular swimming, abnormal mucus secretion, abnormal opercular ventilation, increased air gulping, and changes in skin coloration. Responses to the highest concentrations at weeks two and six were erratic swimming patterns with jerky movements and some fish suspended in vertical positions with their tails pointing downward. This behavior suggested that the fish treated with CYP resorted to increased air gulping possibly to obtain oxygen for the higher metabolic rates and energy required for CYP detoxification. Increased air gulping indicated that CYP might have reduced dissolved oxygen concentrations, which would have adversely affect normal breathing, leading to asphyxiation. In week eight, the subsequent slowed/moderate swimming movement and opercular ventilation were accompanied by a loss of equilibrium and a few fish sinking to the bottom of the tank, becoming motionless, and some dying subsequently. These observations are consistent with Ullah et al. (2015), who report that jumping, abrupt swimming, loss of balance/equilibrium, increased surface activity, and air gulping were behavioral alterations correlated with exposure of *Tor putitora* (Hamilton) fry to acute CYP. These authors also report that behavioral changes were dependent on dosage and the length of exposure. Fish were slow, motionless, and often displayed vertical positions after a prolonged exposure period (Ullah et al. 2015). Many investigators who researched acute and sublethal toxicity of pyrethriodes and other pesticides in the same and different fish species also report similar behavioral responses (Ogueji 1997, Marigoudar et al. 2009, Ezeonyejiaku et al. 2011). Our results indicated that behavioral changes appeared earlier than mortality markers of sublethal toxicity. This phenotypic index was observed to shift depending on dose and exposure. In the 1.1 and 2.1 μg L^{-1} sublethal doses at week eight, moderate (10–50%) and strong (>50%) responses were reported. The skin color of the fish gradually changed to a glossy grey. Pigment cell dispersion in chromatophores that was induced by CYP might have caused this color change. Mucus is secreted as a protective mechanism to shield fish from the impact of toxic agents. However, by hindering ion control and gas exchange in the gill epithelium, increased mucus, particularly in the gills, contributes to asphyxiation. Similar phenotypic observations are

recorded by other investigators (Akinsorotan et al. 2019, Ogueji et al. 2019).

Toxic effects of CYP on serum biochemical parameters

The outcomes of the present study clearly indicated that CYP is highly toxic to *O. niloticus*. Fish metabolize and remove CYP more slowly than do mammals or birds, which possibly explains its greater toxicity to fish (Bradbury and Coast 1989). Due to the lipophilicity of pyrethroids, they have a high rate of gill absorption, which contributes to fish sensitivity to aqueous pyrethroid exposure. Fish seem to be deficient in the enzyme system that hydrolyzes pyrethroid. The main reaction involved in the metabolism of CYP in mice or rats is ester cleavage mainly by the action of carboxyesterase, while metabolism in fish is largely oxidative (Viran et al. 2003). Significant ($P < 0.05$) dose-dependent increases in glucose were recorded during the exposure cycles of weeks six and eight. Increases were not noted in week two. The rise in glucose can be regarded as a manifestation of CYP-induced stress. Increased glucose is a general response of fish to the effects of acute and sublethal pollutants (Luskova et al. 2002, Yaji et al. 2018, Ogueji et al. 2019). Wedemeyer and Wood (1974) reports that high serum glucose levels were caused by physical and chemical stress-induced disorders in the metabolism of carbohydrates. The adrenal tissue is activated by a number of stressors, leading to elevated levels of circulating glucocorticoids and catecholamines (Ogueji et al. 2017). These groups of hormones produce hyperglycemia leading to marked changes in carbohydrate reserves. The observed elevation in serum blood glucose could have been a response to the increased rate of glycogenolysis or gluconeogenesis. Similar findings are reported by Bhanu and Deepak (2015) after *Cyprinus carpio* L. was exposed to CYP. The biphasic protein response in our study showed that CYP elicited significant dose-dependent increases during the exposure cycles of weeks two and six. However, in week eight, protein decreased significantly at CYP concentrations of 1.1 and 2.1 μg L^{-1}. The quantity of serum protein depended on the rate of protein synthesis or on the rate of its degradation. The hyperproteinemia observed might have resulted from increased demand for CYP detoxification or from serum water loss. Hypoproteinemia might have also resulted from the impaired incorporation of amino acids into polypeptide chains (Yadav et al. 2003). Hypoproteinemia was also reported by Bhanu and Deepak (2015) after exposing fish to CYP. Usually, GOT and GPT are found inside cell membranes, mitochondria, and cytoplasm. In these cells, the aggregation or binding of xenobiotics can lead to cell damage and to the release of these enzymes into blood circulation. Consequently, during stress conditions, a significant rise in blood serum transaminases can be expected (Ogueji 2008, Saravanan et al. 2012).

Data taken over two, six, and eight weeks indicated substantial dose-dependent increases in GOT relative to the control. GPT behavior presented as a mixed pattern as CYP resulted in significant dose--dependent decreases over two and six weeks of exposure. However, this pattern was altered by substantial dose-dependent increases in GPT relative to the control in week eight. The significant elevation of GOT activities at the higher sublethal doses of the toxicant implied that faster metabolic activity was induced by the higher sublethal concentrations of CYP in the exposed fish. This could have been attributed to the activation of the hepatic microsomal cytochrome P_{450}-dependent monooxygenase pathway, and since increases in GOT were significantly dose-dependent, this could be interpreted as the antioxidant defenses of *O. niloticus* progressively weakening or being compromised as the sublethal concentration increased that led to oxidative stress and consequent muscle damage especially to the cardiac muscle. Khurshid (2003) proposed that deformities observed in the muscular and nervous systems of chick embryos treated with CYP were caused by the interaction of CYP with the function of acetylcholinesterase neurotransmitters. This author also noted a decrease in chick brain size. Consistent with our findings, Ojutiku et al. (2013) documented

increased ALT (GPT) and AST (GOT) activity in fish after exposure to CYP. Similarly, increased AST (GOT) was recorded in fish treated with concentrations of 1.5 and 2.5 mg L^{-1} of CYP (Montanha et al. 2014). GOT and GPT inhibition was also reported in *C. gariepinus* juveniles exposed to the highest sublethal doses (1.6 x 10^{-3} µg L^{-1}) of lambda-cyhalothrin (pyrethroid). The author maintains that the enzyme activity observed could have been from liver deficiency in the mobilization of enzymes induced by cell death (necrosis, apoptosis, or both).

The liver is the principal center of lipid metabolism (Gopal et al. 1981). The liver is also the key organ in the synthesis and excretion of cholesterol (Ogueji 2008). Therefore, any type of obstruction in the liver, whether intra- or extrahepatic, causes increased total cholesterol levels in the serum. The observed significant dose- and duration-dependent decreases of triglyceride and cholesterol in weeks two and six might have been due to the higher energy demand of *O.niloticus* to achieve positive survival values under the imposed stress of sublethal CYP. However, at week eight and at the maximum sublethal concentrations, triglyceride and cholesterol production increased significantly. This might have been caused by glycogen storage deficiency and hepatic dysfunction induced by prolonged sublethal exposure. Similar decreases in triglycerides and cholesterol were recorded from CYP sublethal stress on *Oncorhynchus mykiss* (Walbaum) (Orun et al. 2014). Some other researchers also report increased values of triglyceride and cholesterol from sublethal CYP exposure (Ojutiku et al. 2013, Bhanu and Deepak 2015). These differences might have been because of the various types of fish used in evaluations. The overall behavior of the serum biochemical parameters of *O. niloticus* during exposure cycles of two, six, and eight weeks to sublethal CYP concentrations indicated that they were consistent. The process whereby animals first go through the alarm phase in the short term, followed by resistance in the medium to long term and eventually fatigue, is consistent with the alarm and resistance phases of the overall stress response.

Conclusion

The findings of the present study showed that CYP is highly toxic to fish. Sublethal concentrations of CYP induced dose- and duration-dependent and biphasic responses that influenced behavioral and biochemical parameters in *O. niloticus*. The results indicated that CYP adversely affected the physiology and health of the fish. Therefore, in order to guard against toxicological effects on non-target species, especially fish in nearby aquatic ecosystems, the application of CYP in agriculture should be controlled.

Disclosure statement. The authors declare no competing interests.

Author contributions. A.Y. performed laboratory experiment, collected the data for behavior and biochemical parameters, analyzed and interpreted the data; E.O.O. performed laboratory experiment, collected the data for behavior and biochemical parameters, analyzed and interpreted the data; J.J.E. wrote the original draft and its final version.

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