***Original Research Article***

# ALTERATIONS IN STEROID SEX HORMONES (17β estradiol and testosterone) OF *Clarias gariepinus* EXPOSED TO DIFFERENT SUB-LETHAL CONCENTRATIONS OF CYPERMETHRIN


**ABSTRACT**

The study focused on the alterations in steroid hormone levels of *Clarias gariepinus*. Fish were exposed to sub-lethal concentrations of cypermethrin over a 28 days period. A total of 200 sub-adults of *C. gariepinus* with mean weight of 55.280 ± 6.281g were used through-out the study. Sub-adults of *C. gariepinus* were exposed to 0.00 ppm, 0.0125 ppm, 0.025 ppm, 0.100 ppm and 0.400 ppm of cypermethrin. The concentration of 17β-Estradiol and testosterone in *C. gariepinus* sub-adults exposed to 0.0125 ppm, 0.025 ppm, 0.100 ppm and 0.400 ppm of the toxicant for 7, 14, 21 and 28 days decreased significantly from the control, with increase in toxicant at $p<0.05$, except for 17β-Estradiol and testosterone in fish exposed to 0.0125 ppm of cypermethrin for 21 days and 7 days. The mean levels of estradiol decreased in 0.400 ppm group compared to the control; from 212.4 ± 3.156 pg/mL to 118.9 ± 9.682 pg/mL (Day 7); 210.5 ± 8.286 pg/mL to 90.70 ± 7.554 pg/mL (Day 14); 131.7 ± 5.652 pg/mL to 80.77 ± 6.882 pg/mL (Day 21) and 177.6 ± 12.25 pg/mL to 52.77 ± 11.08 pg/mL (Day 28). The mean levels of testosterone decreased in 0.400 ppm group compared to the control; from 2.367 ± 0.208 pg/mL to 0.823 ± 0.276 pg/mL (Day 7); 2.700 ± 0.200 pg/mL to 0.466 ± 0.152 pg/mL (Day 14); 3.200 ± 0.300 pg/mL to 0.300 ± 0.100 pg/mL (Day 21) and 2.933 ± 0.251 pg/mL to 0.366 ± 0.208 pg/mL (Day 28). Due to adverse alteration in 17β-Estradiol and testosterone concentration of the test fish, we recommend that the Government sensitizes the farmers properly on the proper use of pesticides and also enforce against excess application of pesticides. More of similar studies should be funded in order to continue monitoring the effects of various pesticides on fishes and the aquatic eco-system at large.



**KEYWORDS:** Alterations, sex steroid hormones, *Clarias gariepinus*, sub-lethal, cypermethrin, concentration


## INTRODUCTION

Pesticides are recognized world-wide as a veritable means of controlling pests, and are highly toxic to other species in the environment at the same time [1]. Presently, there is an increasing concern world over on the indiscriminate use of pesticides, which results in

environmental pollution and toxicity risk to non-target organisms [2]. Cypermethrin is a widely used pyrethroid pesticide, against stored products pests, to control ecto-parasites in both land and aquatic animals [3, 4]. Responses to pyrethroid insecticides by fish are wide ranged, depending on the compound, exposure time, water quality, concentration and species [5]. Non-point source pollutants are mainly transported overland and through the soil by runoff [6]. These pollutants ultimately find their way into groundwater, wetlands, rivers and lakes, and finally, to oceans in the form of sediment and chemical loads carried by rivers [7]. Contamination of water by insecticides is mainly due to intensive agriculture combined with surface runoff and sub-surface drainage, usually within a few weeks after application [8]. Fishes are particularly sensitive to environmental contamination of water. Hence, pollutants such as insecticides may significantly damage certain physiological and biochemical processes when they enter into the organs of fishes [8]. Endocrine disrupting chemicals (EDCs) are defined as chemical substances that alter the normal endocrine function [9], including naturally occurring chemicals such as phytoestrogen or synthetic chemicals such as pesticides, plasticizers, polychlorinated biphenyls (PCBs) and alkylphenolic compounds. EDCs such as methoxychlor pesticide, polychlorinated biphenyls (PCBs) and bisphenol (BPA) antagonizes endogenous hormones such as tamoxifen or disrupt the synthesis and metabolism of hormones or interact with the hormone receptors [10]. In the Ovary, estradiol must be synthesized in sufficient amounts to stimulate the liver to produce vitellogenin. In case of oocyte atresia; the estradiol hormone decrease leads to impaired vitellogenesis [11]. Nile tilapia showed decrease in sex steroid hormones upon exposure to organochlorine pesticide like hexachlorobenzene (HCB) [12]. [13], mentioned that *Oreochromis niloticus* fed with diet incorporated with malathion for 4 month exerted endocrine disrupting effect on males than females by decreasing testosterone hormone; while dimethaote has pronounced effect on females through decreased 17 ß- estradiol hormone. [14] reported that male *Oncorhynchus mykiss* exposed to sublethal concentration of methanote for 30 day showed significant decrease in 17 ß- estradiol. The study was aimed at evaluating the steroid sex hormones alterations in *Clarias gariepinus* sub-adults exposed to different concentrations of cypermethrin.

## MATERIALS AND METHODS

### Collection and transportation of test organisms

*C. gariepinus* sub-adults were collected from the University of Calabar fish farm (Hatchery) along with culture water in a plastic bucket, and transported immediately to

Department of Zoology and Environmental Biology Laboratory, University of Calabar, where they were allowed to acclimate to the laboratory conditions.

**Acclimation and maintenance of study organisms**

In the laboratory, the fish samples were allowed to acclimate to laboratory conditions at room temperature for a period of two (2) weeks during which they were fed with commercial fish feed (coppen) twice daily, at 4% of their body weight.

**Range finding test**

A range finding test was carried-out prior to the commencement of the research, during which the test fish was exposed to a wide range of sub-lethal concentrations of cypermethrin, so as to reveal the most appropriate concentrations for the study.

**Preparation of stock solution**

A stock solution was prepared by adding 990 mL of water to 10 mL of cypermethrin with 96.8% purity and shaken thoroughly to form 1000 mL of stock solution with 10.101 ppm concentration, through which further dilutions into 0.0125, 0.025, 0.100 and 0.400 ppm concentrations were made.

**Experimental design**

Five 44.3 x 29.7 x 15.6 $cm^3$ glass aquarium were used through-out the study. A total of 200 sub-adults of *C. gariepinus* with mean weight of 55.280 ± 6.281g were used through-out the study. Forty sub-adults of the test fish were stocked in each aquarium (i.e per group) containing 25 litres of well treated borehole water in a renewable experiment. Four groups of the fish were exposed differently to 0.0125, 0.025, 0.100 and 0.400 ppm of cypermethrin respectively for 28 days, and the $5^{th}$ group was the control (0 ppm) where no toxicant was introduced. Every 72 hours, the water was renewed, the toxicant introduced again and the fishes fed as well. After 7, 14, 21 and 28 days of exposure, 10 fishes per group were punctured around the cardiac area behind the anal fin and 2 mL of blood samples were collected using a 5 mL syringe into a serum bottle. The collected blood samples were immediately preserved in an ice chest before taking to University of Calabar teaching hospital for analysis of 17β estradiol and testosterone (sex steroid hormones) using enzyme-linked immunosorbent assay (ELISA) kits designed by Diametra S.R.I, Italy.

**Determination of steroid sex hormones**

Sex steroid hormones (testosterone (T) and 17β estradiol (E2)) were extracted using ELISA as described by [15]. Blood of 100 µL was adjusted to 1 mL with milliQ water, after which 5 mL diethyl ether was added. After 45 seconds, samples were spun down for 3 min at 2500 rpm and snap-frozen for 7 seconds in liquid nitrogen. Subsequently the ether phase was evaporated in a warm (40 °C) water bath under a gentle flow of nitrogen gas. The remaining pellets were reconstituted in 500 µL PBS+gelatin and stored at −80 °C until they are analyzed. Sex hormones were determined in triplicate by enzyme-linked immunosorbent assay (ELISA) kits designed by Diametra S.R.I, Italy.

**Statistical analysis**

The data obtained for 17β estradiol and testosterone in the exposed and control fish group were subjected to descriptive statistics (Mean and standard deviation). Analysis of variance (ANOVA) was used to test for the significance of the difference in cypermethrin induced alterations of steroid sex hormones for each group exposed to different concentrations of cypermethrin after 7, 14, 21 and 28 exposure days compared to the control. All analysis was be carried-out using Graphpad Prism version 5 at 0.05 level of significance and at their relevant degree of freedom.

**RESULTS**

**Plasma concentration of estradiol (E2) in *Clarias gariepinus* sub-adults exposed to sub-lethal concentrations of cypermethrin**

The summary of the trend of estradiol concentration in *Clarias gariepinus* exposed to 0.00 ppm (control), 0.0125 ppm, 0.025 ppm, 0.100 ppm and 0.400 ppm of cypermethrin are shown in Figures 1 – 4. The concentration of 17β-Estradiol in *C. gariepinus* sub-adults exposed to 0.0125 ppm, 0.025 ppm, 0.100 ppm and 0.400 ppm of cypermethrin for 7, 14, 21 and 28 days decreased significantly from the control at $p<0.05$ except for the group exposed to 0.0125 ppm concentration of cypermethrin for 21 days (Figures 1 – 4).

The concentration of 17β-Estradiol in *C. gariepinus* sub-adults exposed to 0.00 ppm (control), 0.0125 ppm, 0.025 ppm, 0.100 ppm and 0.400 ppm of cypermethrin for 7, 14, 21 and 28 days decreased with increase in the concentration of the test toxicant (Figures 1 – 4). For the 7 days exposure duration, the highest concentration of 17β-Estradiol in *C. gariepinus* sub-adults was observed in the control group (0.00 ppm), having a mean and standard

deviation of 212.4 ± 3.156 pg/mL, but reduced to 146.7 ± 9.393 pg/mL when exposed to 0.0125 ppm of the toxicant. It further decreased to 140.2 ± 5.448 pg/mL for 0.025 ppm group, 129.5 ± 10.13 pg/mL for 0.100 ppm and 118.8 ± 9.682 pg/mL for 0.400 ppm group (Figure 1).

For the 14 days exposure duration, the highest concentration of 17β-Estradiol in *C. gariepinus* sub-adults was observed in the control group (0.00 ppm), having a mean and standard deviation of 210.5 ± 8.286 pg/mL, but reduced to 151.1 ± 13.510 pg/mL when exposed to 0.0125 ppm cypermethrin. It further decreased to 121.0 ± 11.530 pg/mL for 0.025 ppm group, 102.2 ± 11.090 pg/mL for 0.100 ppm and 90.70 ± 7.553 pg/mL for 0.400 ppm group (Figure 2).

For the 21 days exposure duration, the highest concentration of 17β-Estradiol in *C. gariepinus* sub-adults was observed in the control group (0.00 ppm), having a mean and standard deviation of 131.70 ± 5.652 pg/mL, but reduced to 121.20 ± 5.524 pg/mL when exposed to 0.0125 ppm of the toxicant. It further decreased to 113.40 ± 5.501 pg/mL for 0.025 ppm group, 80.20 ± 6.582 pg/mL for 0.400 ppm and 80.77 ± 6.882 pg/mL for 0.100 ppm group (Figure 3).

For the 28 days exposure duration, the highest concentration of 17β-Estradiol in *C. gariepinus* sub-adults was observed in the control group (0.00 ppm), having a mean and standard deviation of 177.6 ± 12.250 pg/mL, but reduced to 106.5 ± 12.460 pg/mL when exposed to 0.0125 ppm cypermethrin. It further decreased to 85.67 ± 11.910 pg/mL for 0.025 ppm group, 75.20 ± 4.386 pg/mL for 0.100 ppm and 52.77 ± 11.080 pg/mL for 0.400 ppm group (Figure 4).

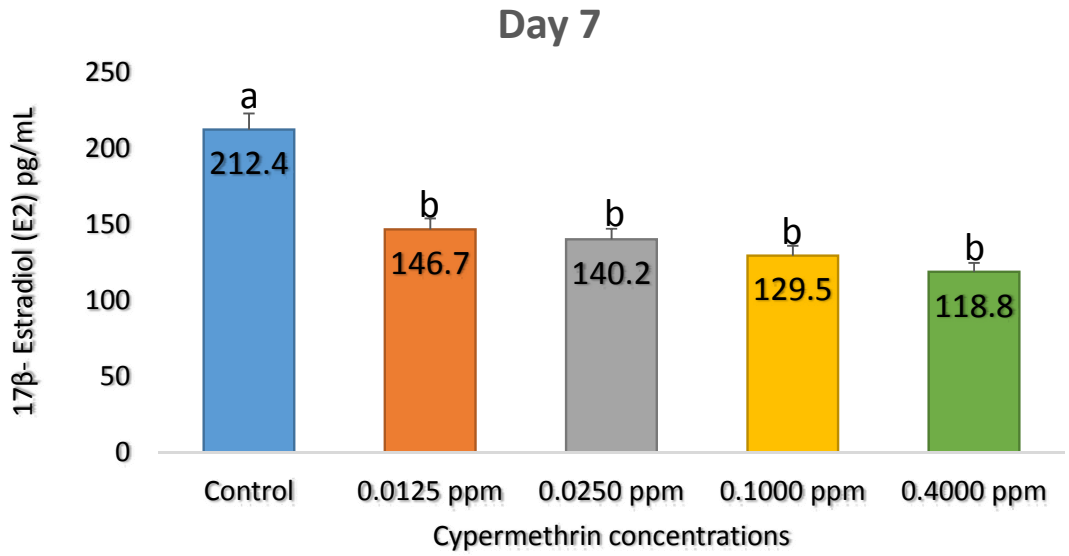


Fig 1: Graph showing the decrease in the concentration of 17β-Estradiol (pg/mL) in *Clarias gariepinus* sub-adults exposed to different concentrations of cypermethrin for 7 days. Concentration of 17β-Estradiol in *C. gariepinus* exposed to 0.0125 ppm, 0.025 ppm, 0.100 ppm and 0.400 ppm of cypermethrin decreased significantly from the control at $p<0.05$. Different alphabets for each group compared to control are significantly different from control at $p<0.05$.

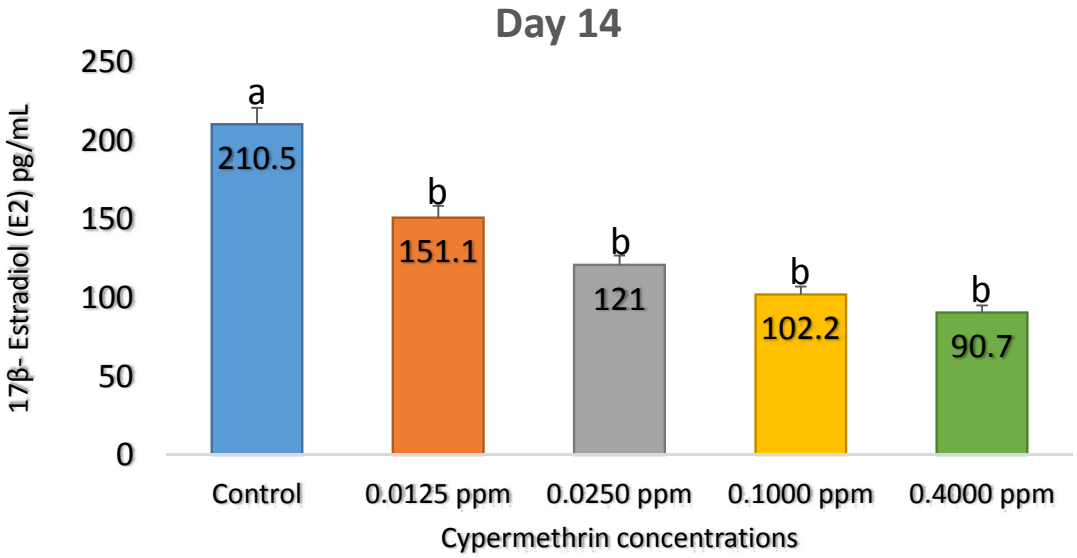


Fig 2: Graph showing the decrease in the concentration of 17β-Estradiol (pg/mL) in *Clarias gariepinus* sub-adults exposed to different concentrations of cypermethrin for 14 days. Concentration of 17β-Estradiol in *C. gariepinus* exposed to 0.0125 ppm, 0.025 ppm, 0.100 ppm and 0.400 ppm of cypermethrin decreased significantly from the control at $p<0.05$. Different alphabets for each group compared to control are significantly different from control at $p<0.05$.

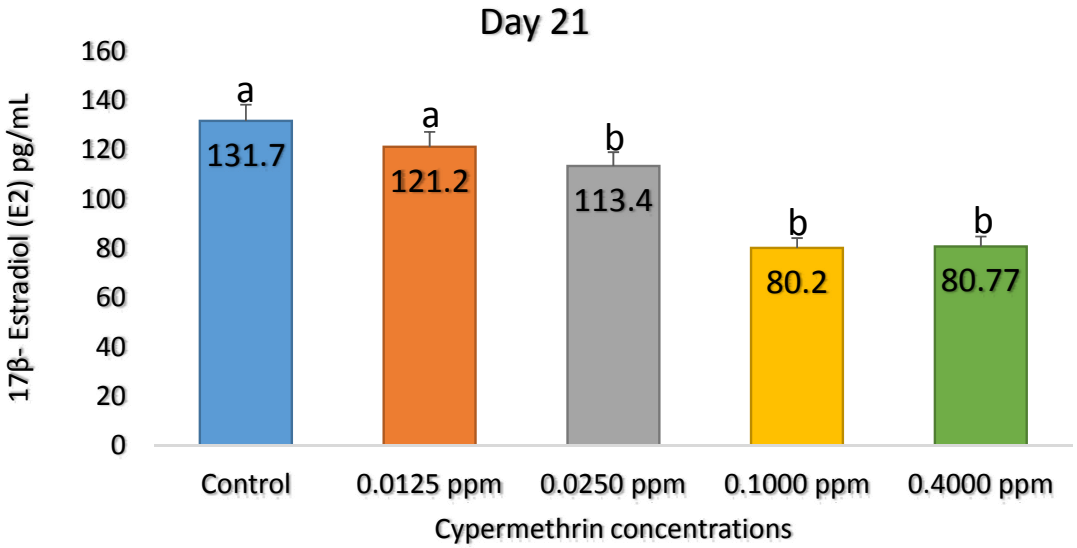


Fig 3: Graph showing the decrease in the concentration of 17β-Estradiol (pg/mL) in *Clarias gariepinus* sub-adults exposed to different concentrations of cypermethrin for 21 days. Concentration of 17β-Estradiol in *C. gariepinus* exposed to 0.025 ppm, 0.100 ppm and 0.400 ppm of cypermethrin decreased significantly from the control at $p<0.05$. Different alphabets for each group compared to control are significantly different from control at $p<0.05$.

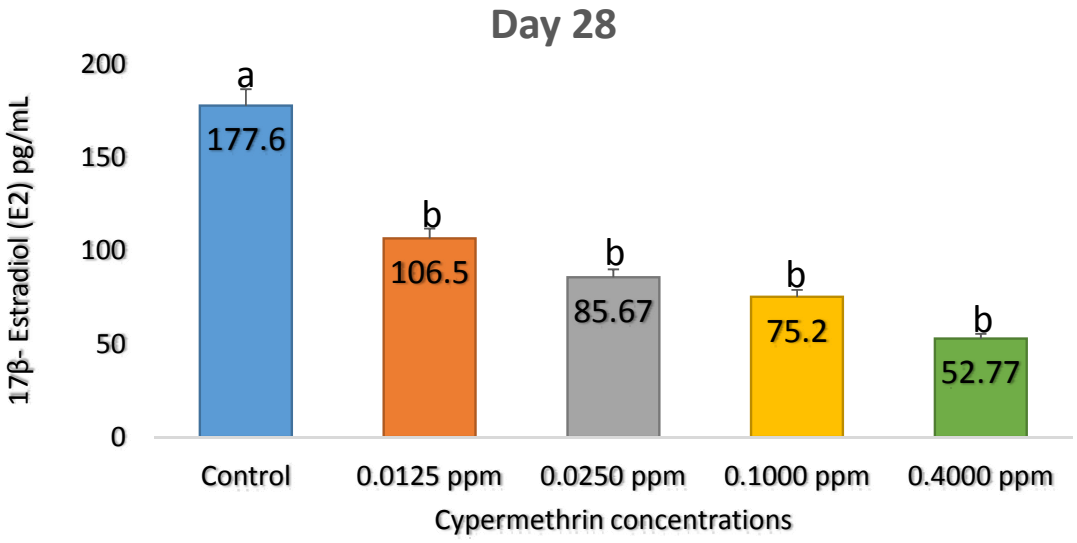


Fig 4: Graph showing the decrease in the concentration of 17β-Estradiol (pg/mL) in *Clarias gariepinus* sub-adults exposed to different concentrations of cypermethrin for 28 days. Concentration of 17β-Estradiol in *C. gariepinus* exposed to 0.0125 ppm, 0.025 ppm, 0.100 ppm and 0.400 ppm of cypermethrin decreased significantly from the control at $p<0.05$. Different alphabets for each group compared to control are significantly different from control at $p<0.05$.

**Plasma concentration of Testosterone (T) in *Clarias gariepinus* sub-adults exposed to sub-lethal concentrations of cypermethrin**

The summary of the testosterone concentration in *Clarias gariepinus* exposed to 0.00 ppm (control), 0.0125 ppm, 0.025 ppm, 0.100 ppm and 0.400 ppm of cypermethrin is shown in Figures 5 – 8. The concentration of testosterone in *C. gariepinus* sub-adults exposed to 0.0125 ppm, 0.025 ppm, 0.100 ppm and 0.400 ppm of cypermethrin for 7, 14, 21 and 28 days decreased significantly from the control at $p<0.05$ except for the group exposed to 0.0125ppm concentration of cypermethrin for 7 days (Figures 5 – 8).

The concentration of testosterone in *C. gariepinus* sub-adults exposed to 0.00 ppm (control), 0.0125 ppm, 0.025 ppm, 0.100 ppm and 0.400 ppm of cypermethrin for 7, 14, 21 and 28 days decreased with increase in the concentration of cypermethrin, except for 0.025 ppm and 0.100 ppm group after 7 days exposure, 0.0125 ppm and 0.025 ppm after 14 days exposure and 0.100 ppm and 0.400 ppm group after 28 days of exposure (Figures 5 – 8). For the 7 days exposure duration, the highest concentration of testosterone in *C. gariepinus* sub-adults was observed in the control group (0.00 ppm), having a mean and standard deviation of 2.367 ± 0.208 pg/mL, but reduced to 1.733 ± 0.351 pg/mL when exposed to 0.0125 ppm cypermethrin. It further decreased to 1.167 ± 0.251 pg/mL and 1.167 ± 0.351 pg/mL for 0.025 ppm and 0.100 ppm group respectively and then 0.823 ± 0.276 pg/mL for the 0.400 ppm group (Figure 5).

For the 14 days exposure duration, the highest concentration of testosterone in *C. gariepinus* sub-adults was observed in the control group (0.00 ppm), having a mean and standard deviation of 2.700 ± 0.200 pg/mL, but reduced to 1.300 ± 0.200 pg/mL when exposed to 0.0125 ppm cypermethrin. It then slightly increased to 1.367 ± 0.208 pg/mL for 0.025 ppm group, but decreased to 0.933 ± 0.251 pg/mL for 0.100 ppm and 0.466 ± 0.152 pg/mL for the 0.400 ppm group (Figure 6).

For the 21 days exposure duration, the highest concentration of testosterone in *C. gariepinus* sub-adults was observed in the control group (0.00 ppm), having a mean and standard deviation of 3.200 ± 0.300 pg/mL, but reduced to 2.167 ± 0.251 pg/mL when exposed to 0.0125 ppm of the toxicant. It further decreased to 1.733 ± 0.351 pg/mL for 0.025 ppm group, 1.067 ± 0.251 pg/mL for 0.100 ppm and 0.300 ± 0.100 pg/mL for the 0.400 ppm group (Figure 7).

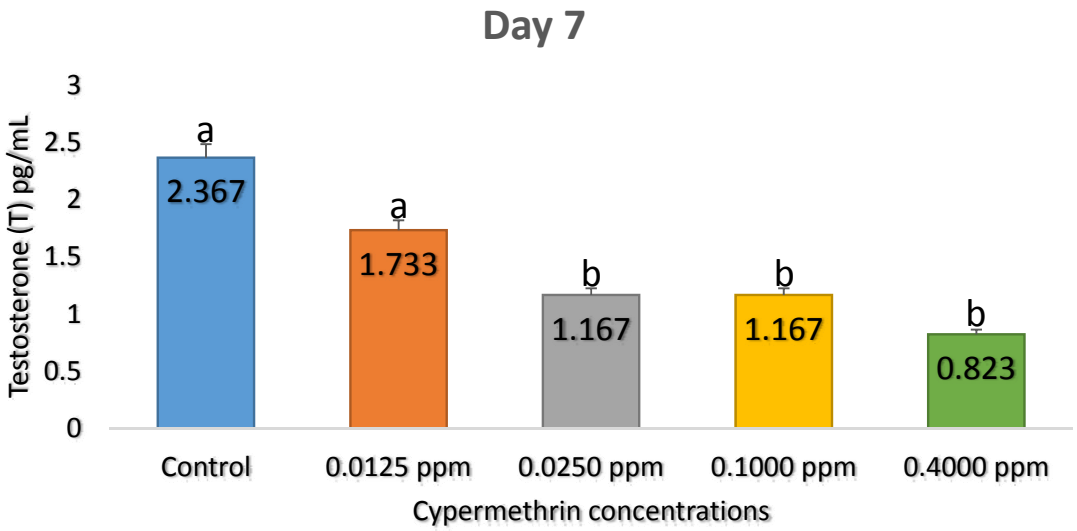


Fig 5: Graph showing the decrease in the concentration of Testosterone (T) (pg/mL) in *C. gariepinus* sub-adults exposed to different concentrations of cypermethrin for 7 days. Concentration of Testosterone (T) in *C. gariepinus* exposed to 0.025 ppm, 0.100 ppm and 0.400 ppm of cypermethrin decreased significantly from the control at $p<0.05$. Different alphabets for each group compared to control are significantly different from control at $p<0.05$.

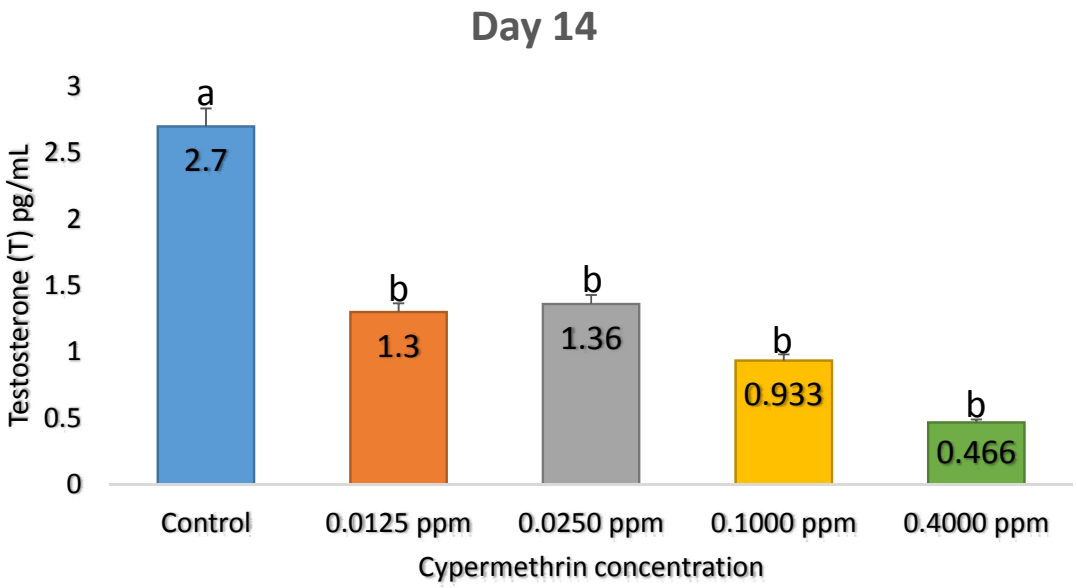


Fig 6: Graph showing the decrease in the concentration of Testosterone (T) (pg/mL) in *C. gariepinus* sub-adults exposed to different concentrations of cypermethrin for 14 days. Concentration of Testosterone (T) in *C. gariepinus* exposed to 0.0125 ppm, 0.025 ppm, 0.100 ppm and 0.400 ppm of cypermethrin decreased significantly from the control at $p<0.05$. Different alphabets for each group compared to control are significantly different from control at $p<0.05$.

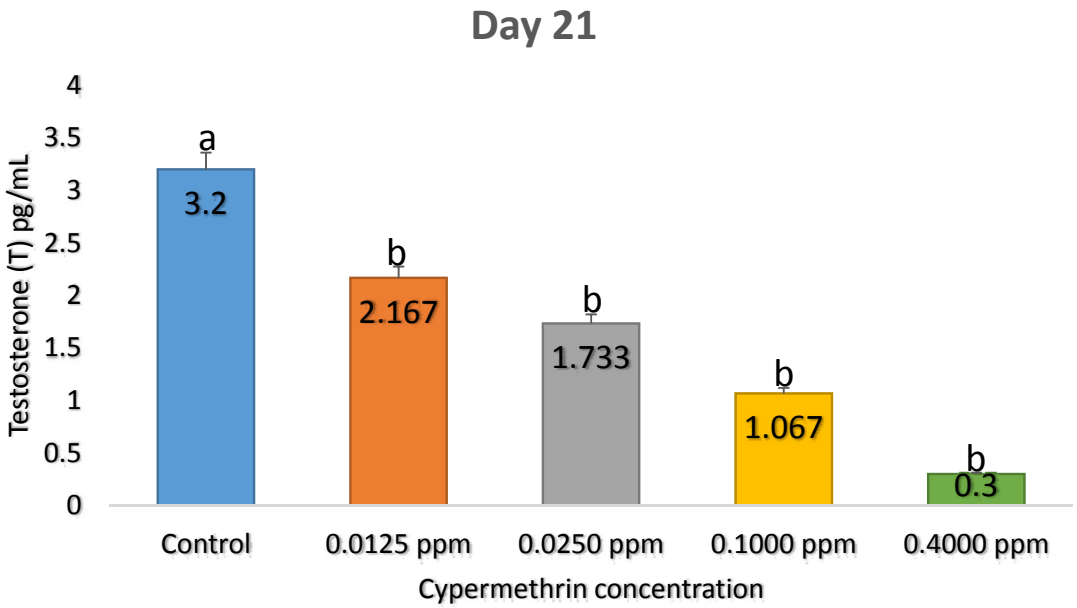


Fig 7: Graph showing the decrease in the concentration of Testosterone (T) (pg/mL) in *Clarias gariepinus* sub-adults exposed to different concentrations of cypermethrin for 21 days. Concentration of Testosterone (T) in *C. gariepinus* exposed to 0.0125 ppm, 0.025 ppm, 0.100 ppm and 0.400 ppm of cypermethrin decreased significantly from the control at $p<0.05$. Different alphabets for each group compared to control are significantly different from control at $p<0.05$.

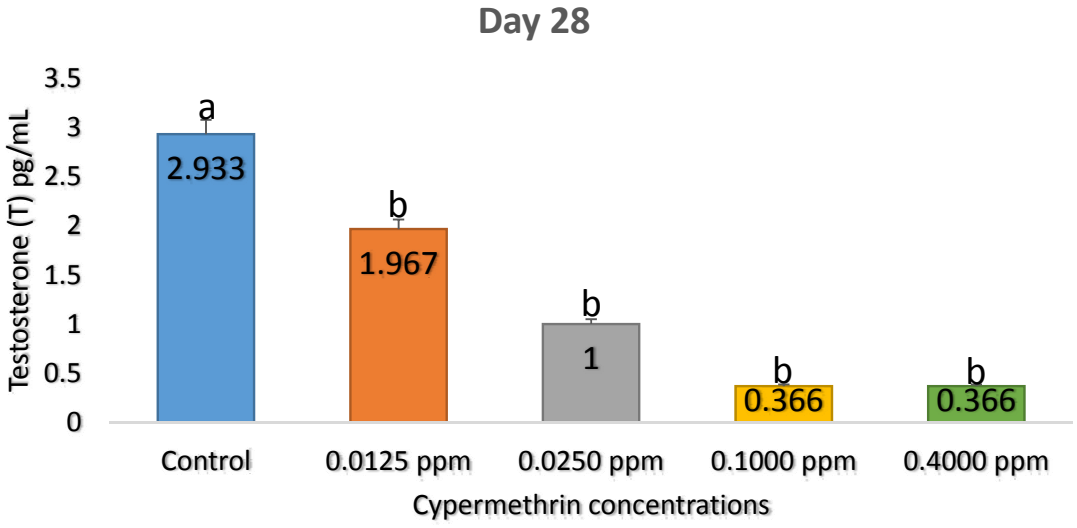


Fig 8: Graph showing the decrease in the concentration of Testosterone (T) (pg/ml) in *Clarias gariepinus* sub-adults exposed to different concentrations of cypermethrin for 21 days. Concentration of Testosterone (T) in *C. gariepinus* exposed to 0.0125 ppm, 0.025 ppm, 0.100 ppm and 0.400 ppm of cypermethrin decreased significantly from the control at $p<0.05$. Different alphabets for each group compared to control are significantly different from control at $p<0.05$.

For the 28 days exposure duration, the highest concentration of testosterone in *C. gariepinus* sub-adults was observed in the control group (0.00 ppm), having a mean and standard deviation of 2.933 ± 0.251 pg/mL, but reduced to 1.967 ± 0.251 pg/mL when exposed to 0.0125 ppm of the toxicant. It further decreased to 1.000 ± 0.200 pg/mL for 0.025 ppm group and then to 0.366 ± 0.115 pg/mL and 0.366 ± 0.208 pg/mL for 0.100 ppm and 0.400 ppm concentration groups respectively (Figure 8).

**DISCUSSION**

Fishes are often used to assess the health of aquatic environment and physiological changes occurring as a result of pollution in an aquatic environment [16]. The physiological alterations caused by chemicals like; cypermethrin have been found to affect the endocrine system. Endocrine disrupting chemicals (EDCs) like; cypermethrin act mainly by interfering with natural hormones due to their strong potential to bind to estrogen or androgen receptors, causing impairment of fish fecundity, semen quality, hatchability and survivability of fish [17]. Sex steroid hormones, vitellogenin, organosomatic index and histopathological changes are considered as biomarker tools for assessing the endocrine disrupting effect of pesticides on fish. Steroid hormones are one of the several hormones that influence fish reproduction, and as such the major androgens produced by testicular tissue differ from one fish species to another.

This study revealed a decrease in the concentration of 17β-Estradiol and testosterone in *Clarias gariepinus* sub-adults with increase in the concentration of the cypermethrin for most fish group after 7, 14, 21 and 28 days exposure period. Also, the concentration of 17β-Estradiol and testosterone in *C. gariepinus* sub-adults exposed to different sub-lethal concentrations of cypermethrin decreased significantly from the control for the 7, 14, 21 and 28 days exposure period, except for the 0.0125 ppm groups after 21 days and 7 days of exposure respectively, causing vitellogenesis. This result corroborated with the findings of [18]; who reported decrease in testosterone and estradiol levels in *Oreochromis niloticus* compared to the control when exposed to Chlorpyrifos; [12], who reported that Nile tilapia showed decrease in sex steroid hormones upon exposure to organochlorine pesticide like; hexachlorobenzene (HCB); [13], who reported more endocrine disrupting effect on males than females through decreasing testosterone hormone for *O. niloticus* exposed to malathion and [11], who reported that exposure to pesticide causes decrease in estradiol level compared to the control, leading to impaired vitellogenesis, since estradiol must be synthesized in

sufficient amount to stimulate production of vitellogenin in the liver. The decrease in the concentration of estradiol and testosterone in the sub-adults of *C. gariepinus* when exposed to sub-lethal concentrations of cypermethrin compared to the control group could be due to the impairment of the secretion of the 11- ketotestosterone from the sertoli [19], 17ß- estradiol from the follicular cells [20], molecular mimickery of the hormones which stimulates a negative feedback in gonadotrophin secretion, suppression of the synthesis of endogenous estrogen [21] and increase in the steroid metabolizing enzymes activities [22]. The reduction in the concentration of testosterone and estradiol in *C. gariepinus* exposed to the pesticide could also be due to interference with the production of free cholesterol, sex hormone precursor which would have been converted into testosterone (T) and estradiol (E2) by the enzyme aromatase [23].

The decrease in the testosterone and estradiol concentration in *C. gariepinus* exposed to the toxicant, was contrary to the findings of [24] who reported an increase in both sex hormones, and this could be due to the difference in duration of exposure, frequency of exposure, toxicity of the chemical, age of fish, season, sex and species. Abnormal and behavioural responses were also observed on exposure of fish to cypermethrin, causing in-active swimming, loss of equilibrium, vertical hanging in water, less feeding, erratic swimming and air gulping at water surface. Appearance of mucous covering on the gills, along with the change in the colour of the lamellae were also observed as part of the adverse physiological effects of cypermethrin on the test fish.

Consequently, the result reveals alterations in the rate of spermatogenesis and oogenesis due to apoptosis, leading to the disturbance in normal hormone physiology and unexpected pressures on endocrine homeostasis.

**CONCLUSION**

In conclusion, the study revealed sub-lethal toxic effect of cypermethrin on levels of steroid hormone production, by decreasing the production of 17β-Estradiol and testosterone in the fish, subsequently leading to impaired vitellogenesis and disruption of endocrine glands in general. The study also revealed that the decrease in the concentration of 17β-Estradiol and testosterone in fish was concentration dependent, except for few groups. Also, important roles of sex steroids (androgens and estrogens) in the regulation of reproduction and other physiological processes was revealed. As a result of the adverse alteration in 17β-

Estradiol and testosterone of the test fish, we recommend that Government sensitizes the farmers properly on the proper use of pesticides, so as to ensure its controlled use.

## ETHICAL CONSIDERATION

The authors ensured that all the ethical and other basic principles underlying behavior and advancing welfare for the use of animals in research, including handling, relevant laws and regulations were considered before proceeding with the research. Bio-ethical clearance for use of animals for laboratory studies was issued to the authors before carrying-out the research.

## COMPETING INTEREST

Authors have declared that no competing interests exist, but rather the research was a collective effort of all the authors.

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