Multiple Biomarker Responses in Female Catfish (Clarias Gariepinus) Exposed to Acetaminophen

Nosakhare Osazee Erhumwunse (✉ nosakhare.erhumwunse@uniben.edu)
University of Benin  https://orcid.org/0000-0003-2725-0571

Isioma Tongo
University of Benin

Lawrence Ikechukwu Ezemonye
University of Benin

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Abstract

Acetaminophen is one of the most commonly detected Analgesics and pain killer drug in freshwater environments. This study evaluated the possible multi-toxic effects of environmentally relevant concentrations (15.5, 25.5, 35.5 and 45.5µg/L) of acetaminophen in *Clarias gariepinus* fish exposed for 28 days using multiple biomarkers. Hepatosomatic index (HIS) and condition factor (K) of acetaminophen–exposed group were not different from the control. The superoxide dismutase (SOD) activity increased significantly at 15.5 and 35.5µg/L and Catalase (CAT) activity in all acetaminophen-exposed groups barely showed an upward trend. The concentration of glutathione S-transferase (GST) activities was not different from the control. Glutathione peroxidase (GPx) activities increased at all concentrations when compared to the control group. There were general inhibitions of Acetylcholinesterase (AChE) activities in all exposed groups including the control. Total antioxidant capacity (TAC) increased significantly at 25.5 and 45.5µg/L and Interluekin-6 (IL-6) showed non-significant increases in all exposed concentrations. Acetaminophen exposure caused non-significant increases in the activities of C reactive protein (CRP). White blood cells (WBC) and lymphocytes (LYM counts) were significant reduced. Acetaminophen induced significant changes in hormones of the hypothalamic-pituitary-gonad (HPG) axis (17β-Estradiol and Testosterone) and vitellogenin (Vtg) synthesis at 45.5µg/L. Histopathological alterations in the liver was evident of apoptotic hyperplasia, sinusoidal congestion and necrosis of the hepatocytes and was concentration dependent. Acetaminophen exposure to the fish gills enhanced the fusion and shortening of some filaments, hyperplasia of the epithelia gills cells, aneurism, congestion and epithelia rupture of the gills. Gonad examination showed acetaminophen exposure triggered the occurrence of intersex in 25.5, 35.5 and 45.5µg/L. The collaborative biomarkers used in this study showed the multiple impacts of acetaminophen on the physiology of *C. gariepinus*. Multivariate statistical analysis indicated that fish in the control groups exhibited a distinctly response from the acetaminophen-exposed fish and that over 95% of the biomarkers significantly contribute to discriminate between the acetaminophen-exposed fish and the control group. Our research provides evidence supporting the use of multiple-biomarker approach to evaluate the health status of *C. gariepinus* in future studies.

Highlights

- Oxidative stress markers (SOD, TAC) and immune-modulatory markers showed good and early signs of Acetaminophen (APAP) exposure.
- High levels of IL-6 and CRP confirm APAP-induced liver damage.
- The histopathological evaluation of exposed organs provides evidence of hepatotoxicity and gonadal alterations as a result of exposure to acetaminophen.
- Alterations in Testosterone and 17β-Estradiol indicate possible signs of reproductive failure.

1.0 Introduction
Acetaminophen (APAP) (paracetamol) is the most used analgesic and antipyretic medication in the world that has been used alone and effectively supplemented with other medications to treat colds and pain (Guiloski et al., 2017). It is undoubtedly one of the most threatening substances applied in medicine, producing over 458 numbers of death in the United State of America due to acute liver failure (ALF) (Lee 2004, Brune et al., 2014). The precise mechanism of action responsible for the analgesic effect of acetaminophen is still unknown despite its long history of use (Jozwiak-Bebenista and Nowak, 2014; Guiloski et al., 2017). Acetaminophen and its metabolites are endlessly introduced into aquatic habitat mainly from clinics and domestic wastewater, personal use and waste discharges from drug production sites (Ebele et al. 2017; Zur et al., 2018). APAP reach the environment mainly excreted in an unaltered or slightly metabolized form (Zur et al. 2018) and due to the elevated and effective released rate, APAP and its associated metabolites (APAP, acetaminophen; APAP gluc, acetaminophen glucuronide; APAP-cys, acetaminophen cysteine; NAPQI, N-acetyl-p-benzoquinone imine) is readily detected in treated wastewater, groundwater and rivers at varying concentrations (Mazaleuska et al., 2015; Folarin et al., 2019; Ebele et al., 2020).

Over the years, the demand for the consumption of acetaminophen has increased, (Bloughz and Wu 2011, Gulmez et al., 2013; Moore and Moore, 2016) consequently leading to its high concentration of 1020 ng g\(^{-1}\)-155.6 µg/L in our environment (Al-Mashaqbeh et al., 2019). APAP can react with hypochlorite during the water disinfection phase, “leading to the occurrence of two extremely toxic compounds: 1, 4-benzoquinone and hepatotoxic N-acetyl-p-benzoquinone imine that can impact aquatic organisms” (Bedner and MacCrehan, 2006; David and Pancharatna, 2009; Sung et al., 2014; Guiloski et al., 2017).

Several reports has shown that Acetaminophen can act as potential endocrine disruptor, induce oxidative stress, hepatotoxic, neurotoxic and disrupt physiological process in exposed aquatic organism; hindering the usual embryonic development, growth, behaviour and survival of Daino rerio and Clarias gariepinus larvae (David and Pancharatna 2009, Erhunmwunse et al., 2021). Sublethal concentrations altered swimming behaviour, pigmentation and energy balance in Phalloceros harpagos (Matus et al., 2018). Sublethal concentrations induced endocrine disruption and altered the liver structure in male fish Rhamdia quelen (Guiloski et al., 2017); induced oxidative stress in Clarias gariepinus (Folarin et al., 2018; Yakubu et al., 2020).

The need to assess the multiple effects associated with acetaminophen in a single exposed-species is important and requires a holistic use of multiple biomarkers comprising haematological, biochemical, and histopathological indices. The collaborative use of series of corresponding biomarkers that can specify exposure and measure the effects of acetaminophen on the exposed fish enables more extensive and combined evaluation of biochemical and cellular impacts caused by the acetaminophen (Linde-Arias et al., 2008; Cazenave et al., 2009). Hence, the multi-biomarker techniques have attracted significant attention in ecology and toxicological research and have been used in both un-controlled and controlled experimental studies (Bacchetta et al., 2014, Javed et al., 2017). This study was therefore aimed at assessing multi-biomarkers responses in Clarias gariepinus exposed to environmental concentrations of acetaminophen.
2.0 Materials And Methods

2.1. Fish

Post-Juvenile *C. gariepinus* (*n* = 150, 20.25±1.76cm (length) and 91.34±2.95g (weight)) were obtained from the Faculty of Agriculture Farm Project and transferred to the aquatic trial section of Ecotoxicology Laboratory, University of Benin and conformed to laboratory conditions for 14 days in 250-L capacity aquaria (26 ± 1°C). The fish were kept under natural light/dark cycle and fed daily with Durante fish feed. The use of fish for this study was authorized by the Animal Experimentation Ethics Committee of the University of Benin, Faculty of Life Sciences and all protocols followed the International Guidelines for Animal Use.

2.2 Chemicals Purchase

Acetaminophen (≥ 98% purity, CAS Number 103-90-2), was purchased from Sigma-Aldrich (St. Louis, MO, USA). Fish Vitellogenin ELISA (Cat. No. E0020Fi) was obtained from Biotechnology Laboratory (China), Interleukin 6 from CUSABIO Tech LLC (Catalog No: CSB-E13258Fh TX, USA) High Sensitivity C-Reactive Protein (CRP) ELISA (Cat. No. CR375C), SOD, Glutathione Peroxidase, Total Antioxidant Capacity, Acetylcholinesterase, Catalase Assay Kits were obtained from Calbiotech Inc. (CA, USA).

2.3. Experimental Design

Experimental tests were carried out in 60-L glass aquaria in a static-renewal state under OECD guideline No. 203; 229 and 230 (OECD, 1992, 2009, 2012) and APHA (1998). The experiments were carried out in 1:1 light-dark cycles in 24hrs timeline. The water quality parameters of the experimental water were analysed by the methods of APHA (1998) Temperature had an average value 26.10±0.34°C, 6.50±1.01 (pH), 15.40±0.10µs/cm (Electrical conductivity), 6.20±0.34 mg/L (dissolved oxygen), 10.20±0.72mg/L (total hardness), 0.03±1.50 mg/L (salinity), 9.40±2.40 mg/L (total suspended solid). Fish (*n* = 7 per treatment) were exposed to five nominal concentrations of acetaminophen (15.5, 25.5, 35.5, and 45.5µg/L µg/L). A range-finding test was carried out by the procedure employed by Omitoyin et al. (1995); Rahman et al. (2002), and was used to establish the concentration of acetaminophen for the definite test. A logarithmic dilution series was used for the range-finding assay. For the definitive test, stock solutions of the required concentrations were prepared. 35.5g acetaminophen powder was dissolved in 1 L of distilled water. The stock was then diluted serially and the required concentrations were used for the definitive test and prepared every 48hrs.

2.4. Measurement Of Biomarkers

Endpoints used to assess sublethal toxicity of acetaminophen include alterations in physiological condition indices (condition factor (K), hepatosomatic index (HSI) and gonadosomatic index (GSI)),
Haematological alterations (Full Blood count), reproductive alterations (testosterone, 17β-Estradiol and plasma vitellogenin (VTG), histopathological alterations (gills, liver and gonad (for post-juveniles) and alterations in oxidative stress and antioxidant defence markers (SOD/Catalase, GST, Glutathione peroxidase (GPx), Acetylcholinesterase, Total Antioxidant Capacity(TAC)) and immunological (IL-6, CRP). After 28 days of exposure, the sh were anaesthetized with benzocaine 1%. Blood was taken by the perforating the genital opening with a heparin-coated 21 gauge ×0.5in needle, attached to a 2 ml syringe of steroid hormones. The fish were put down by medullar section. The liver, gills and gonads were used for histopathological analysis.

2.4.1 Biomarkers Of Oxidative Stress

2.4.1.1 Superoxide dismutase activity (SOD)

SOD activity in the supernatant was evaluated by the procedure of Magnani et al. 2000. SOD activity was measured by its ability to stimulate pyrogallol autoxidation. The pyrogallol autoxidation in the case of EDTA at pH 8.2 is 50%. Blood was centrifuged for 10 min at 3500 rpm to discharge the plasma. The centrifuged erythrocytes were made up of 2.0 ml of distilled water (40°C) and disparate for 15 min. The assay was performed by applying 50 µl of supernatant to a clean tank. 1ml of SOD assay buffer was applied to the cuvette, followed by 1ml of SOD chromogenic solution added to the cuvette and combined for 1min. Absorbance was assessed at 420 nm immediately and after 2 min. The activity is expressed in SOD units per mg of tissue protein.

2.4.1.2 Catalase Activity

Catalase was determined by measuring the H$_2$O$_2$ substrate remaining after the decomposition of hydrogen peroxide to H$_2$O and O following the method of Ding et al., 2000. The assay reaction was performed at room temperature (25°C). 25ul blood agar was introduced into a test tube followed by 75ul assay buffer. Another 25ul of substrate was added, mixed and incubated for 15 minutes, and then 825ul of the stop solution was introduce into the mixture and disparate. 10ul aliquot of the mixture was removed and introduced into another test tube. 1ml of the chromogen reagent was added and mixed thoroughly and allowed to cool for 15 minutes for colour development. The resultant colour is read at 520nm.

2.4.1.3. Glutathione Peroxidase Activity (Gpx)

Glutathione peroxidase activity in plasma was determined by the oxidation of Glutathione (GSH) by cumene hydroperoxides. The oxidized glutathione was reduced in the presence of glutathione reductase (GR) and NADPH. In this reaction the NADPH is oxidized to NADP$^+$ and the absorbance was measured at 340nm. The enzyme activity was expressed in µmol H$_2$O$_2$ min$^{-1}$ mg of protein$^{-1}$. 
2.4.1.4. Total Antioxidant Capacity (TAC)

TAC was determined by the reducing potential of the plasma to covert Cu$^{2+}$ to Cu$^+$ and the ion absorbance was measured at 450nm. Fish blood samples were centrifuge at 3000 x g for 12 minutes at 4°C and the plasma and aliquot were stored at -70°C. 10µL serum and 190 µL Dilution buffer were introduced to a 96-well microplate and read at 450nm. 50µL of chromogen were added to each well and incubated for 5minutes (25°C) and absorbance read for the second time at 450nm.

2.4.2 Immunotoxicity Biomarkers

2.4.2.1. Determination of C reactive protein (CRP)

CRP was determined using a quantitative sandwich enzyme immunoassay technique (ELISA) (Kindmark, 1972). Before assay, all reagents and samples were maintained at 25°C. Blood samples of the acetaminophen-exposed sh and the control were diluted 1:1000 by adding 5µl of samples to obtain 495µl of sample diluent. 10µl of Standard, diluted samples and control were added to the wells; 100 µl HRP-Conjugate solution was introduced into every well except blank wells; Air bubbles were drawn out from the liquid by tapping the holder, and mix properly and incubated for 60 minutes at 25°C. The liquid was removed from the wells and wells were rinsed 3times with 300ul of 1x buffer and plotted on absorbance paper. A solution of TB substrate was added to the well and placed in the oven for 15 minutes. Absorbance was read at 450 nm within 15 minutes after adding Stop Solution.

2.4.2.2 Il-6 Activity

Plasma IL-6 intensity was calculated using ELISA with a reactivity of 12.5 pg/mL using the accessible IL-6 ELISA fish kit as instructed by the producer.

2.4.3 Reproductive Biomarkers/ Steroid Hormones

2.4.3.1 Determination of Vitellogenin Elisa

Assessment of plasma VTG was determined using ELISA kit (Bioassay Technology Laboratory, China). The plates were supplied pre-coated with VTG fish antibodies. 40µl fish plasma (VTG) introduced into the plate binds to the antibodies coated with the well. 50µl biotinylated fish VTG is added and binds to the sample VTG followed by the addition of 50µl Streptavidin-HRP to the solution and incubated for 60 minutes at 37°C. Plates were rinsed 5 times with buffer followed by the addition of 50µl of substrate A and B simultaneously and incubated for 10 minutes at 37°C. 50µl of stop solution added and observed a colour change from blue to yellow. The optical density was read at 450nm.
2.4.3.2 17β-estradiol And Testosterone

The levels of testosterone (T) and 17β-estradiol (E2) in test fish were determined using Enzyme Immunoassay (EIA) kits (Cayman Chemical Company, USA). Blood samples were obtained using method employed by Alohan et al, (2014). Plasma samples were taken after the blood samples were spun at 7,000 g for 10 minutes. The plasma samples were aliquoted and kept at -20°C until they were analyzed. Methylene chloride was used to extract E2 from the aliquot of plasma, whereas diethyl ether was used to extract T. Under a nitrogen stream, the extracts were evaporated. By vortexing, the dry extract was reconstituted in 500 mL Enzyme Immuno-Assay (EIA) buffer. E2 and T were made in duplicate and measured. EIA measured the E2 and T levels using the method of the Azuadi et al. (2013) and absorbance was read at 405 nm.

2.4.4 Neurotransmitter Biomarker

2.4.4.1 Determination of Acetylcholinesterase

Sample preparation of blood sample was diluted 40-fold in Assay Buffer. 10ul of samples was introduced into a 96 well microplate. The blank well contained 200up of distilled water. 160ul assay buffer was introduced to the sample wells followed by 20ul ACHE chromogen. The absorbance was read after 2 minutes at 413NM and the absorbance was recorded. 10ul of ACHE Substrate was added, disparate and let to stand for 10min. The absorbance was read again at 412nm and the initial absorbance was recorded and values of acetylcholine calculated.

2.4.5. Haematological Biomarkers

Haematology indices were determined following the method of Banaee et al., 2008; “Red cell indices: mean corpuscular volume (mcv:fl/cell),and mean corpuscular haemoglobin (mch: pg /cell) were calculated from RBC (10^6/mm^3), Ht (%) and Hb (g/dl) according to Lee et al., (1998)”

2.4.6. Histopathological Biomarkers

Histological slides of studied organs were developed following the method of Erhunmwunse et al. (2014). “Organs were harvested and rinsed in saltwater to take out the blood and fixed in Bouins’s fixative for 24 hours. The organs were dehydrated in a graded series of alcohol (ethanol) and fixed in paraffin wax (M.P. 58-680c). Alterations caused by acetaminophen exposure in the organs were analyzed and photographed under photomicroscope along with control group. Tissue samples were sent to the Faculty of Basic Medicine, Department of Anatomy, University of Benin, for final histological processing and preparation (HE staining) for light microscopy analysis”.
2.7. Statistical Analysis

Statistical calculations were carried out with GraphPad Prism 8 software. Data were presented as mean ± Standard error of mean (SEM). The parameters were calculated using one-way ANOVA followed by the Dunnett’s multi-comparison test was used to denote the significant differences between acetaminophen concentrations and the control treatment. Differences were considered significant at $p \leq 0.05$. Due to the large number of biomarkers analysed, Principal component analysis (PCA) was used to correlate Acetaminophen-exposed fish with the control group with the responses of biomarkers.

3.0. Result

3.1. Physiological Condition Indices

The Fish morphometric indices (K and HIS) were not significantly affected ($p > 0.05$) by the exposure to acetaminophen (Fig. 1a & b), but lead to slight increases in all exposed groups as the condition factors of the fishes increased by 24.14, 6.89, 3.45 and 1.72% at 15.50, 25.50, 35.50 and 45.50 µg/L respectively compared to the control. In HIS, an increase of 6.0, 30.0, 53.0 and 42.0% was observed at 15.50, 25.50, 35.50 and 45.50µg/L (Fig. 1). The gonadosomatic index of the fish were significantly reduced ($p < 0.05$) by acetaminophen exposure compared to the control groups. The reductions were 51.90, 80.76, 73.99 and 78.09% at 15.50, 25.50, 35.50 and 45.50µg/L (Fig. 1).

3.2. Oxidative Stress Biomarkers

The SOD enzyme activities were significantly increased ($p < 0.05$) in all acetaminophen-exposed groups compared to the control. The significant differential increases of 189.29, 89.29, 153.57 and 71.43% were observed at 15.50, 25.50, 35.50 and 45.50µg/L respectively (Fig. 2A).

Acetaminophen exposure caused an increase in Catalase activity (Fig. 2B). The Catalase activity increased with an increasing concentration of acetaminophen with the highest concentration of 45.5µg/L having the highest Catalase activity in all plasma studied. The differential increase in Catalase activity was in the sequence of 45.5>15.50>25.50>35.50µg/L. An increase of 23.25, 21.37, 14.90 and 25.49% at 15.50, 25.50, 35.50 and 45.50µg/L were observed compared to the control.

The measured GST activities of fish are shown in (Fig. 2C). Acetaminophen exposures did not cause any noticeable change ($p > 0.05$) in GST activities and were found to fluctuate as GST activities were reduced in 15.50 and 35.50µg/L by 21.00 and 14.28% and increased in 25.50 and 45.50µg/L by 28.57 and 50.0% increase respectively (Fig. 2C).

Fish exposed to acetaminophen revealed a remarkable reduction in GPx activity compared to the control (Fig. 2D). The differential decrease in the GPx did not show a dose-response pattern with 74.00, 36.42, 70.71 and 68.57% increases in 15.50, 25.50, 35.50 and 45.50µg/L compared to the control.
Acetaminophen caused an increase in TAC activities in all exposed fish compared to the control. Significant increases were observed at 25.5 and 45.5µg/L (Fig. 3B). An increase of 122.72, 404.54, 309.09 and 400% at 15.50, 25.50, 35.50 and 45.50µg/L was recorded.

### 3.3. Neurotransmitter

In term of AChE induction, there were no significant increases \((p>0.05)\) in the acetaminophen treated groups compared to the control. Extraneous increases in AChE activities in 15.50, 35.50 and 45.50 were 08.09, 11.51 and 7.80% respectively and a non-significant reduction of 06.38% in 25.50µg/L (Fig. 3A).

### 3.4. Immunological Markers

Immunological analysis of IL-6 demonstrated that the exposure to acetaminophen caused non-significant increases of 21.02, 6.25, 34.09 and 21.02% at 15.50, 25.50, 35.50 and 45.50µg/L (Fig. 3C). C reactive protein activities were significantly increased \((p<0.0001)\) in all acetaminophen exposed groups compared to the control (Fig. 3D). The increases were 136.59, 131.71, 136.59 and 136.59% at 15.50, 25.50, 35.50 and 45.50µg/L respectively.

### 3.5. Haematological Parameters

There were increases in WBC's of acetaminophen-exposed fish relative to the control. Increases of 101.28, 113.86, 112.57 and 61.00% were reported in WBC at different concentrations of 15.50, 25.50, 35.50 and 45.50µg/L (Fig. 4a). RBC, HGB and HCT fluctuated in the acetaminophen-exposed groups as opposed to the control. The HGB responses were found to fluctuate by 118.41, 11.64, 71.98 and 27.93% at different concentrations of 15.50, 25.50, 53.50 and 45.50µg/L, respectively. An increase of 60 and 26.65% at 15.50 and 35.50µg/L and a decrease of 35.09 and 5.81% at 25.50 and 45.50µg/L were documented in HCT. Acetaminophen caused increases in MCV, MCH in all exposed groups while the MCHC count was not affected. PLT increased in all exposed fish is predicted to decrease at 15.50µg/L. Lymphocyte increased by 81.49, 90.34 and 48.03% in fish exposed to 15.50, 35.50 and 43.50 µg/L, while decreases in the community exposed to 25.50 µg/L were observed.

### 3.6. Reproductive Biomarkers

Only fish subjected to 43.5µg/L acetaminophen showed statistically significant increased \((p<0.05)\) VTG levels than control fish. The levels of testosterone were significantly reduced in all acetaminophen-exposed fish by 19.71, 13.84 and 37.12% in 25.50, 35.50 and 45.50µg/L (Fig. 4). Acetaminophen exposure caused 17β-Estradiol increases in all exposed group compare to the control. Fish from 35.5µg/L acetaminophen-exposed group had significantly lower level of 17β-Estradiol \((p < 0.05)\) compared to
controls. The levels of 17β-Estradiol in exposed fish increased by 41.58, 33.22, 30.82 and 31.74% at 15.50, 25.50, 35.50 and 45.50µg/L compared to the control respectively.

3.7.0. Histopathology

3.7.1. Gills

Acetaminophen exposure caused histopathological alterations in the gill of exposed fish (Fig. 5). The common changes observed on the gills of acetaminophen-exposed fish were huge elevation of the lamellar epithelium, edema of the lamellae, blood congestion, exfoliated epithelium of lamellae, an increase of mucosal cells, lamellar disorganization, hypertrophy and hyperplasia of the epithelial cell which resulted in incomplete merging of the secondary lamellae and a reduced water space at 45.50, 35.50 and 25.50µg/L. Hemorrhagic telangiectasia, fatty lobes, biliary atresia, hepatic fibrosis, congestion, Kupfer cells, apoptotic hepatocytes, portal tract edema, single-cell necrosis, sclerosis cholangitis, biliary cirrhosis, coagulative necrosis, inflammatory cells infiltration characterized the gills of fish exposed to 15.50µg/L.

3.7.2. Liver

The alterations in the liver of acetaminophen-exposed C. gariepinus are shown in Fig. 6. Fish in the control group showed normal hepatic structure and parenchyma. The most frequent alterations found in the liver of acetaminophen-exposed fish were single-cell necrosis, hyperplasia, sinusoidal congestion, degeneration of the hepatocyte, sclerosis cholangitis, coagulative necrosis, focal necrosis, thickening of the tunica adventitia and portal tract edema. The severity of these alterations increased with increasing concentration. Hepatocellular carcinoma, dilation of central vein, cavernous hemangioma, biliary cirrhosis, hepatic steatosis, focal nodular hyperplasia, dilation of central vein, hepatic oedema, Portal tract edema, thickening of the tunica adventitia characterized the liver of fish exposed to 35.50 and 25.50µg/L (Fig. 6). Hemorrhagic telangiectasia, biliary atresia, apoptotic hepatocytes, portal tract edema, biliary cirrhosis, coagulative necrosis, inflammatory cells infiltration were common alterations observed in 15.50µg/L.

3.7.3. Gonads

Acetaminophen exposure caused histological changes in the gonads of the acetaminophen-exposed catfish (Fig. 7). Ovarian examination of catfish and the corresponding changes were observed at various concentrations of acetaminophen. There were no reported alterations in the gonad of fish gonads in the control group (Figure 7a) as the photomicrograph showed oocytes with perinucleolar phase (Primary growth oocytes). Acetaminophen exposure caused several alterations in the exposed groups compared to controls, degenerative nucleus; degenerative follicles were noticed in the ovarian structure in the experimental groups. In the 15.50µg/L group, histopathological alterations were observed at minimum.
degree and include disintegrative follicles, necrosis and the destruction of muscles bundles (Figure 7b). An increase in the primary oocyte numbers and yellow patches was observed at 25.50µg/L (Figure 7c). The female gonad exposed to 35.50µg/L showed alterations which include increased atretic follicles, vascuolarized oocytes, and necrosis of the oocytes, the appearance of fat deposit (Yellow patches), degenerative nucleus and ovarian hyperplasia (Figure 7d). Disintegration in ovarian structures was observed for the highest concentration. (Figure 7d). Others include large occurrence of atretic follicles, yellow patches, necrosis and vascuolarized oocytes.

3.7.4 Multivariate analysis

The results of the PCA of the fish exposed to vary concentrations of acetaminophen plotted against the fish in the control are shown in Figure 9. There was a clear distinction between the fish in the control on the left site of the plot and the Acetaminophen-exposed fish on the right side of the plot. Figure 9 also reveals that the pattern of most biomarkers in acetaminophen-exposed-fish is strongly correlated with increasing concentration.

4.0 Discussion

4.1 Effect of Acetaminophen on Physiological Condition Indices and Oxidative stress biomarkers

Exposure to acetaminophen at environmentally relevant concentrations had distinct effects in female post-juvenile *Clarias gariepinus*. Morphometric analyses demonstrated that the HIS and K of the acetaminophen-exposed fish did show any significant change and this was similar to the report of Stancova et al. (2014) who observed no changes in growth parameters of Tench (*Tinca tinca*) exposed to a combination of ibuprofen, diclofenac, and carbamazepine for 35 days. Acetaminophen significantly reduced gonadosomatic index of exposed female *C. gariepinus* and agrees with the report of Mills et al. 2011 who showed a significant decrease in the gonadosomatic index of marine fish Cunner (*Tautogolabrus adspersus*) exposed to an anticancer drug, Anastrozole.

The biotransformation pathway of APAP has showed that the toxicity is as a result of the formation of the very active metabolite N-acetyl-p-benzo-quinone Imine (NAPQ1) by cytochrome P450s (Macherey, 2015; Ramos-Tavar and Muriel, 2019) when consumed at a non-pharmacologic doses. At the high doses, the pathways for the transformation of APAA (sulfation and glucuronidation) becomes saturated and a portion of APAP is excreted in the parent form leading to excess NAPQ1 which further reacts with other protein groups to form protein adducts (McGill et al., 2013) leading to oxidative stress and mitochondrial dysfunction resulting in the production of free radicals.

The increased SOD enzyme activities must have been triggered by the presence of acetaminophen which produced reactive oxygen species (ROS) (Halliwell and Gutteridge, 1999). As the levels of ROS increases, the biological system creates a first-line defence mechanism by altering the behaviours of SOD (Roberts
and Oris, 2004; Bagnyukova et al., 2006). Increased SOD activity is an indication of increased antioxidant status that attempts to neutralize the ROS effect (Kurutas, 2016). The elevated CAT activity in our study might be as a result of the functioning of the defence mechanism, which counteracts the oxidative stress induced by acetaminophen metabolism in exposed catfish (Li et al., 2010; Shukla et al., 2017). Acetaminophens have been reported to disturb the redox status of the organism (Trachootham et al. 2008) and CAT level is an excellent marker of protein oxidation and lipid peroxidation (Bohn, 2019).

Several authors hitherto detailed the significance of GST, GPx, and GSH in averting cellular destruction (Livingstone, 2001; Guiloski et al., 2015). The depletions in GPx activities observed in acetaminophen-exposed fish may have been a sign of the induced distress in the fish and agrees with the findings of Guiloski et al. (2015) who reported a decline in GPx activities in gonads of H. malabaricus exposed to dexamethasone.

GST is a phase II detoxifying enzyme that catalyzes the conjugation of the reduced form of glutathione to xenobiotics for detoxification (Stancova et al., 2017). GST is known to be the first line of defence preventing oxidative stress damage, decomposition of superoxide radicals and hydrogen peroxide before interacting to form reactive hydroxyl, which has many unfavourable biological consequences when present in high concentrations (Kaur et al., 2017). The increase in GST enzyme activity in exposed catfish may have represented the occurrence of a defensive mechanism to prevent the impact of acetaminophen, as indicated by Sayeed et al. (2003).

The increase in TAC activity in exposed fish could have been triggered by the oxidative stress in the exposed fish, which may be due to an increase in antioxidant activity employing a cellular defensive mechanism to achieve homeostasis and, in some way, adaptation in the management of antioxidant stress (Kohen et al., 2000, Castillo et al., 2006). A similar report of an increase in TAC activity is comparable to the report of Mantle et al. (2000) who observed increase in TAC activity in human administered with perioperative anaesthetics dopamine, propofol, dobutamine and noradrenaline.

4.2 Immunological Biomarkers Alteration

C-reactive protein (CRP) is an acute inflammatory protein that can increase its activity at infection or inflammation sites (Sproston and Ashworth, 2018). While it is mainly generated by hepatocytes in the liver, it can also be produced by smooth muscle cells, macrophages, and endothelial cells. CRP is involved in inflammatory processes and host responses to infection, such as the complement pathway, apoptosis, phagocytosis, and the development of cytokines (IL), specifically interleukin-6 and tumor necrosis factor-α (Sproston and Ashworth, 2018; Pathak and Agrawal 2019).

It has been established in previous paragraph that in the metabolism of APAP, abundant NAPQI use up the glutathione (GSH) of the cell and bond the proteins covalently, leading to mitochondrial dysfunction, necrosis of the hepatocyte (Fig. 7) and eventually the death of cells (Hinson et al., 2010; McGill et al., 2012). When parenchymal hepatocytes are impaired, innate immunity plays a role in the development
and enhancement of liver injury triggered by APAP. The induction is marked by the release of pro-
inflammatory cytokines and chemokines by activated Kupffer cells (resident hepatic macrophages),
which leads to signal propagation marked by the facilitation of neutrophil adhesion and transmigration
into the hepatic vasculature, which aggravates liver injury (Liu et al., 2004; Liao et al., 2016).

Increased C-reactive protein (CRP) in all acetaminophen-exposed fish may have resulted from the
entrance of pro-inflammatory cytokines into the circulatory system of the exposed fish due to the
presence of acetaminophen (Gani et al. 2009). Our study revealed that acetaminophen induces the
expression of CRP in exposed fish, which may be an indication that the synthesis of the CRP is part of the
host defence reaction to the toxic effect of Acetaminophen. In the recent past, the innate immune system
of fish has drawn attention and it’s considered to be crucial in primary defence and adaptive immunity in
fish (Whyte, 2007). Acetaminophen exposure caused elevated hepatocyte injuries as seen in the
histopathological analysis of the liver and the increased level of CRP shows the protective function
(Sproston and Ashworth 2018). Similarly, Ghosh et al. (1993) and Kodama et al. (2004) documented a
rise in CRP activity in fish exposed to Carbaryl and formalin.

Interleukin-6 (IL-6) is one of the most pleiotropic cytokines due to its function in both innate and adaptive
immune responses and other physiological processes (Varela et al. 2012). Our study showed increased
IL-6 activity in the acetaminophen-exposed fish. This increase may be an indication that IL-6 returned the
exposed fishes to a homeostatic condition and it has been stated that IL-6 runs to regulate the magnitude
of tissue inflammatory responses (Gabay, 2006, Hong et al., 2013). Interleukin (IL)-6 is developed at the
site of inflammation and plays a significant role in the acute phase response as defined by a variety of
diagnostic and biological characteristics, such as the production of acute-phase proteins (Gabay, 2006).
IL-6 is an inflammatory cytokine that is freed from monocytes, lymphocytes at the sites of tissue injury
and may have been freed from the severe liver, gill injuries observed in this study (Gani et al., 2009).
Several authors (Kopf et al. 1994, Xing et al. 1994, Romani et al., 1996, Ruzek et al., 1997, Gabay, 2006)
have noted that in chronic disorder, usually evidenced by immune stressors such as chronic intracellular
infection and tumours, IL-6 not only induce acute phase reactions but also perform an important role in
producing cellular immune responses to affected cells and mucosal humoral responses to re-infection.

4.4 Neurotransmitter Biomarker

The inhibition of AChE in all Acetaminophen-exposed fish suggests an adverse consequence in
cholinergic neurotransmission, and probably in nervous and neuromuscular function (Ribeiro et al.,
2017). The normalization of the enzyme activity in the exposed fish compared to the control can be due
to the compensatory response of the fish cell to the toxic effects induced by acetaminophen exposures.
This study has shown the susceptibility of AChE activities in plasma of acetaminophen-exposed C.
gariepinus (Mdegela et al., 2010). These results indicate that C. gariepinus is a model organism for
pollutants monitoring and evaluation (Mdegela et al., 2006) and the activity of AChE in C. gariepinus is a
prospective diagnostic tool for environmental pollution assessment of anticholinesterases.
4.5 Haematological Indices

There were increased responses in most haematological parameters of exposed fish except MCV and MCH after the 28 days exposure period. Acetaminophen-exposed fish showed an increase in WBC counts which indicate immune and protective response (Saravanan et al., 2011) to the presence of acetaminophen. The increased WBC counts show that *C. gariepinus* develop a protective mechanism to control the toxic stress caused by acetaminophen exposure (Mohammod Mostakim et al., 2015). Similarly, ibuprofen and clofibric acid increased WBCs in exposed *C. carpio* and *Cirrhinus mrigala* (Saravanan et al., 2011, 2012). Again, the observed increased WBC may reveal physiological and immunological (inflammatory) challenges in response to the toxic impact of the acetaminophen (Alimba et al., 2019). The observed reduction in RBC counts at 25.50µg/L might be inhibition of RBC production by acetaminophen. The reduction in RBC counts in fish may show the anaemic state of the fish under stress conditions (Sigbojorn and Tatjana, 2015). Acetaminophen exposure resulted in increased HGB, HCT, Lymphocyte, platelets counts and this may be ascribed to the stimulation of the hematopoietic system of the fish, caused by the exposure.

4.6 Reproductive Markers

Vitellogenin (VTG) is a known biomarker for the confirmation of endocrine activity in fish and it is used in numerous OECD test guidelines (TG) to detect the activities of chemicals on hormonal pathways (Baumann et al. 2019). Acetaminophen-induced VTG in female fish with increasing concentration. Induction of VTG has been reported in fish following exposure to pharmaceuticals, heavy metals and pesticides (Versonnen et al. 2003, Hook et al. 2014). The increased VTG activities in all exposed catfish correspond to the fact that Estradiol induces high levels of VTG (Versonnen et al. 2003). This study agrees with the report of Hong et al. (2007) who stated that NSAID diclofenac increased VTG expression levels in male Japanese medaka following 96 hours of exposure to concentrations as low as 1µg/L. This study thus suggests that analgesic drugs (acetaminophen) are capable of endocrine disruption via estrogenic effects. Hepatotoxic chemicals, on the other hand, have been shown to dramatically disrupt VTG synthesis in trout liver cells in an in vitro research (Miller et al., 1999, Ayobaha et al., 2020). Baumann et al., 2020 reported similar significant increase (*p*<0.05) in vtg in female zebrafish exposed to acetaminophen. Furthermore, Wheeler and Coady, 2016 reported that if liver damage worsens, the functional capacity to process hormones may be diminished, potentially leading to higher circulating estrogen and vitellogenin levels. Again VTG production can be influenced not just by endocrine-related pathways, but also by non-endocrine-mediated mechanisms. Because VTG is formed in hepatocytes, hepatotoxicity, or toxicant-induced deterioration of liver structure and function, could affect VTG as a biomarker (Baumann et al., 2020).

Endocrine-disrupting compounds (EDCs) disrupt hormonal pathways in some organisms, negatively influencing reproductive performance (Martin and Voulvoulis, 2009). There was an anti-androgenic effect observed in all acetaminophen-exposed fish as a result of the increased aromatase activity which
facilitated the transformation of Testosterone to Estradiol and therefore leads to a reduction of Testosterone (Fenske and Segner 2004, Baumann, 2012). There was a concentration-dependent increase in 17β-estradiol with the corresponding reduction of T. These alterations in Estradiol concentration and aromatase activity are significant as they can lead to the alterations in reproduction (Flippin et al. 2007; Han et al. 2010). Similarly, the report of Guiloski et al. (2015) showed a reduction in testosterone levels when male fish *Hoplias malabaricus* were exposed to anti-inflammatory drugs diclofenac and dexamethasone. Guiloski et al. (2017) revealed that the anti-androgenic impact of acetaminophen in male fish *Rhamdia quelen*, as the reduction in testosterone levels and a corresponding increase in the volume of 17β-estradiol were found in fish exposed to higher concentrations of acetaminophen.

### 4.7 Histopathology Biomarkers

Histological changes are associated with complex biochemical and physiological responses to any stressor (Lushchak et al. 2018). Tissue histology is seen as a sign of exposure to contaminants and is an essential apparatus for assessing pollution levels, especially for short-term exposure and prolonged effects (Mostakim et al. 2015). Changes in fish tissue histology have been commonly used, both in controlled experiments and field studies to assess fish health (Mela et al. 2007, Erhunmwunse et al. 2014, Alimba et al. 2015, Alimba et al. 2019).

“Injury to gill tissue can impede the gas exchange capacity of gill and cause respiratory disturbances, ion modulation and osmoregulation dysfunction, and inefficiency in the removal of waste nitrogen metabolites in exposed fish” (Nero et al. 2006; Cengiz and Unlu, 2006; Velmurugan et al. 2007; Banaee, 2013). The gill morphology showed there were disruptions in the lamellar epithelium, gill hyperplasia, cell proliferation, and lamellar fusion in the gills of acetaminophen-exposed fish. Besides, secondary lamellae often had vascular lesions, such as aneurysms, caused by cell disruption and haemorrhage. These findings indicate a reduced respiratory potential and the development of osmotic imbalances due to acetaminophen-induced stress. Lamellar fusion occurred as a result of the excessive proliferation of filament epithelial cells and is an innate defensive mechanism to shield the lamellar epithelium from direct interaction with acetaminophen (Flores-Lopes and Thomaz 2011; Hadi and Alwan 2012). “The fusion and hyperplasia of gill lamellae can be caused by the effects of acetaminophen altering glycoprotein in the cell-covered mucus, influencing the negative charge of the epithelium and favouring adhesion to adjacent lamellae” (Hadi and Alwan 2012; Malarvizhi et al., 2012). The presence of lamellar telangiectasia in the gills of exposed fishes must have resulted from the break-up of elongated bodies and capillaries under the effect of acetaminophen which leads to the accumulation of erythrocytes in the distal section of the secondary lamellae (Randi et al. 1996; Hadi and Alwan 2012). “Changes such as epithelial lifting, hyperplasia and hypertrophy of the epithelial cells, besides partial fusion of some secondary lamellae, are examples of defence mechanisms, since; in general, these result in the increase of the distance between the external milieu and the blood” (Hadi and Alwan 2012). Hoeger et al. (2005) reported severe histopathological changes in the gill of diclofenac treated fish *Salmo trutta*. 
The liver is primarily involved in metabolism, detoxification, storage, and removal of xenobiotics and their metabolites (Mela et al. 2007, Alimba et al. 2019). Liver morphology of acetaminophen-exposed fish showed several alterations which were concentration-dependent. Degenerated vacuolar, Kuffer cells activation, hepatocellular necrosis, single-cell necrosis, coagulation necrosis, focal necrosis, necrosis with inflammatory cells, spotty necrosis, necrosis characterized by hyperplasia with loss of cellular details and necrosis of hepatocytes characterized the liver. Matos et al. 2007; Sepici-Dinçel et al. 2009; Banaee, 2013 “reported similar histopathological alterations in the liver tissue of O. niloticus and C. carpio exposed to sub-lethal concentrations of carbaryl and cyuthrin, respectively”. Acetaminophen cause liver injury in animals in large concentrations leading to increased liver enzymes (Kumar et al. 2004, Guiloski et al. 2017).

Gonad histological analysis proved useful in understanding and evaluating the impacts of possible endocrine-disrupting compounds on aquatic organisms (Leino et al. 2005). Acetaminophen-exposed female fish showed several altered ovarian structure with patches of degenerative follicles, follicular atresia, necrosis and degenerative nucleus. Atresia follicles were lower in lower concentrations of acetaminophen exposure. Increased follicle atresia in the ovaries of estrogen-exposed fish have been reported by some authors (Länge et al., 2001; Metcalfe et al., 2001; Zillioux et al., 2001; Kidd et al., 2007; Kaptaner and Ünal, 2011). Acetaminophen caused degenerative nucleus and follicle, necrosis atresia, eccentric nucleus, ovarian cell hyperplasia and destructive ovarian wall. Similarly some classes of phthalates have been shown to cause atresia of follicle, degenerative follicle in both human and fish (Hannon and Flaws 2015).

4.8 Multivariate Statistical Analysis

Cazenave et al., 2009 highlighted the significant use of arrays of biomarkers when assessing the biological effects in polluted environments, since one marker may not show the true status of exposed organisms. Beliaeff and Burgeot (2002) have stressed the need for researchers to carefully select suitable fusion of markers that can yield data about universal detrimental environmental effects. The statistical analyses of our results distinctly confirm that the use of a single biomarker analysis was not enough to detect alteration induced by acetaminophen in the in exposed fish. Nevertheless, the assessment of multiple-biomarkers in physiological, biochemical, oxidative, reproductive, immunological and neurological activities in the C. gariepinus validate to visibly differentiate between the control groups and the acetaminophen-exposed groups. Multivariate statistical analysis (Figure 9) indicated that fish in the control groups exhibited a distinctly response from the acetaminophen-exposed fish and that over 95% of the biomarkers significantly contribute to discriminate between the acetaminophen-exposed fish and the control group. Figure 9 denotes that acetaminophen-exposed fish may have undergone several stresses, possibly due to exposure to different acetaminophen concentrations.

Conclusion
The consideration of multiple biomarkers in fish for the evaluation of single pharmaceuticals will be a useful tool in assessing the effects of pharmaceuticals in exposed fish. The multiple biomarker approach in this study gives an insight to the capacity of APAP to cause multiple changes from cellular to organismal level and reduce the possibility of biasness in single biomarker evaluation. The multiple biomarkers provided more information on the overall health status of the APAP-exposed fish which include the induction of oxidative stress, activation of the immune response system, alteration of the haematological profile and oxidative damage in many tissues. The studied organs (gills, liver and gonads) provided better information on the hepatotoxicity, neurotoxicity and immunomodulatory effect of APAP at varying concentrations. Our study has showed that multiple biomarkers evaluation revealed consistent evidence on the health status of the fish and provides better vigorous information obvious in justifying the multiple effects of APAP previously misjudged and documented. The multivariate analysis further justifies the use of multiple biomarkers in potential unknown impact of APAP in exposed fish. We therefore recommend that the antioxidant activities (SOD, TAC), immunomodulators (IL-6, C-RP), haematological (WBC, LYM, PLT), intersex in gonad and hormones of the HPGH axis (testosterone and 17β-Estradiol) may be used as indicators for the evaluation of fish fitness and toxicological impacts of PPCPs.

Declarations

Ethical Approval

The use of fish for this study was authorized by the Animal Experimentation Ethics Committee of the University of Benin, Faculty of Life Sciences and all protocols followed the International Guidelines for Animal Use.

Consent to Participate

Not applicable

Consent to Publish

Not applicable

Authors Contribution

Erhunmwunse Nosakhare Osazee, Isioma Tongo, Lawrence Ezemonye: Conceptualization, Methodology, Software. Erhunmwunse Nosakhare Osazee, Isioma Tongo: Data curation, Writing- Original draft preparation. Nosakhare Osazee Erhunmwunse: Visualization, Investigation. Lawrence Ezemonye, Isioma Tongo: Supervision: Isioma Tongo, Erhunmwunse Nosakhare Osazee: Software, Validation: Erhunmwunse Nosakhare Osazee, Isioma Tongo: Writing- Reviewing and Editing.

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**Competing Interests**

The authors declare no competing interests.

**Availability of data and materials**

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

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Figures

![Figure 1](image)

**Figure 1**

Box and whisker plots of Acetaminophen-exposed fish and control. Plot shows Min to Max.) (a) Condition factor (K), (b) HSI (c) GSI (Mean ± SE) * p ≤ 0.05 (n=7).
Figure 2

Box and whisker plots of Acetaminophen-exposed fish and control group. Plot shows Minimum to Maximum values of oxidative biomarkers responses in catfish (A) SOD (B) CAT (C) GST activity (D) GPx. Values represent mean ± standard error of each concentration. Asterisk (*) shows the significant difference between the exposed group and the control *p<0.05, **p<0.01 (n=7).
Figure 3

Box and whisker plots of Acetaminophen-exposed fish and control group. A plots show Minimum to Maximum values (A) AChE (B) TAC (C) IL-6 (D) CRP. Asterisk (*) shows the significant difference in the exposed groups and the control *p<0.05, ***p<0.001. (n=7).
Figure 4

Box and whisker plots of Acetaminophen-exposed fish and control group. Plot shows Minimum to Maximum values on reproductive biomarkers (A) VTG (B) E2(C) T. Values represent the mean ± standard error of each concentration. Asterisk (*) shows the significant difference in the exposed groups and the control *p<0.05, **p<0.001, ***p<0.001. (n=7).
Figure 5

The effect of APAP on Haematological response in catfish shown by Column bar chat. Values represent mean ± standard error of each concentration. Asterisk (*) indicate significant difference from the control *p<0.05, **p<0.01; ***p<0.001. (n=7). (A) WBC. (B) RBC (C) HGB (D) HCT (E) MCV (F) MCV (G) (H) LYM.
Figure 6

Histopathological responses following exposure of C. gariepinus to APAP for 28 days. In gill, the alterations were concentration-dependent and they include loss of secondary lamella (LSL), filament disorganization (FD), the fusion of some filaments (FF), shortening of some filaments (SSL), filament disorganization, Necrosis (N), Hypertrophy and hyperplasia of the epithelial cells of gills (H & HP),
Hyperplasia of epithelia Cells (HEC), Hypertrophy and hyperplasia of the epithelia gills cells (H & H), Aneurysm (A), Congestion (C). (A=Control, B=15.50, C=25.50, D=35.50, E=45.50µg/L). Scale: 200µm

Figure 7

Histological sections of the liver exposed to APAP shows alterations which include: Single-cell necrosis (SCN), Sinusoidal congestion (SC), Sclerosis cholangitis (SC), Coagulative necrosis (CN), Focal necrosis (FN), Portal tract oedema (PTE), Hepatocellular carcinoma (HC), Degeneration of hepatocytes (DH),
Necrosis with inflammatory cells (NIC), Dilation of central vein (DCV), Hepatic oedema, Portal tract edema (PTE), Vacuolation of hepatocytes (VH), Thickening of the tunica adventitia (TTA), Hemorrhagic telangiectasia (HT), Fatty lobes (FL), Biliary atresia (BA), Hepatic fibrosis (HF), biliary cirrhosis (BC), Coagulative necrosis (CN), Inflammatory cells infiltration (ICI). A=Control, B=15.50, C=25.50, D=35.50, E=45.50µg/L). Scale: 200µm

Figure 8
Sectional examination of ovarian tissue of female catfish exposed to acetaminophen for 28 days. The ovaries were mainly composed of follicles in different stages of development. A- (Control) with perinucleolar phase oocytes. The photomicrograph of (B) 15.5 µg/L (C) 25.5 µg/L (D) 35.5 µg/L (E) 45.5 µg/L Acetaminophen. The photomicrograph showed: Necrosis (N), Yellow patches (YP), Improper muscle bundle (IMB), Vasculocariaized oocytes (VOs), Atretic follicles (AF), Eccentric nucleus (EN), Ovarian cell hyperplasia (OCH), Degenerative nucleus (DN), Post-ovulatory follicle (POF), Degenerative follicle (DF), Ovarian wall (OW). Scale = 200 pixel.

Figure 9

Principal Component Analysis (PCA) indicating difference in the biomarkers responses in fish exposed to varying concentrations of Acetaminophen and the control group. The proportions of data is explained by component 1 and 2.

Supplementary Files

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