Innate Immune Response and Growth Performanceof *Heterobranchus longifilis* Catfish Juveniles Following Dietary Aflatoxin B1 Exposure.

Adeyemo, B. T.[1,*]; Enefe, G. N.[2]; Tanimomo, B.[1]; Ezekwesili, A.[1];

1. Department of Veterinary Pathology, Faculty of Veterinary Medicine,
University of Abuja, PMB 117 Abuja, Nigeria.
2. Department of Veterinary Physiology and Biochemistry, Faculty of
Veterinary Medicine, University of Abuja, PMB 117 Abuja, Nigeria.

ABSTRACT

We evaluated the effects of dietary aflatoxin B1 (AFB_1) exposure on the immune response and the growth performance of Heterobranchus longifilis catfish; and determined the maximum tolerable limits for AFB_1 in H. longifilis. Five experimental isocaloric and isonitrogenous diets amended with varied concentrations of AFB_1 [Diet A (2.0 µg $AFB_1.kg^{-1}$); Diet B (7.3 µg $AFB_1.kg^{-1}$); Diet C (17.6 µg $AFB_1.kg^{-1}$); Diet D (48.0 µg $AFB_1.kg^{-1}$) and Diet E (93.0 µg $AFB_1.kg^{-1}$)] were fed to 450 juvenile H. longifilis in five groups of 15 separate plastic tanks for a period or 56 days. At time points day 7, 14, 28 and day 56, five fish were randomly sampled per tank and assessed for innate immunity and growth response. Fish fed diets amended with AFB_1 exhibited significant reduction (P < 0.05) in the serum lysozyme and epidermal mucus lysozyme activity, epidermal mucus alkaline phosphatase activity and in the phagocyte respiratory burst activity. These parameters decreased with increasing concentrations and duration of dietary AFB_1(P< 0.05). Dietary AFB_1 caused significant reductions in the growth performance of juvenile H. longifilis marked by reductions in the percentage gain in length, reduction in percentage gain in weight and a reduction in the specific growth rate. Based on these results, juvenile H. longifilis catfish appears to tolerate diets containing up to 17.6 µg. Kg^{-1} AFB_1in diets.

Key Words: Aflatoxin B1; Innate Immunity; Lysozyme; Respiratory burst; Growth Performance; Heterobranchus longifilis.

*Corresponding Author

Email: bolade.adeyemo@uniabuja.edu.ng

Adeyemo B. T. et al / J. Vet Biomed. Sci.

44

INTRODUCTION

The global production of cultured aquatic organisms (fish, shrimps, clams and oysters) more than doubled in weight and value in the 1990s [1]. These products represents a significant source of animal protein [2] [3]. However, as aquaculture continue to expand and intensify both its reliance and its impact on the ocean fisheries are likely to increase; with a concomitant depletion in the landing of wild-caught fish, which is commonly used as a source of protein in aqua-feed formulations [3] [4]. To mitigate this problem and support the sustainability of the growths recorded in aquaculture,plant based protein sources has been introduced as supplementations or replacements of fishmeal in aqua-feeds[5] [6] [7].

Heterobranchus longifilis is a clariid catfish commonly farmed in earthen pond culture systems in the West African sub region. *H. longifilis* is cultivated based on a diet consisting of groundnut cake and or soybean cake as supplementation/replacement for fishmeal[8]. These plant-based by-products of various agro-industrial processes have been reported to contain varying concentrations of mycotoxins like aflatoxin B1 [9] [10] [11].

Aflatoxin B1 (AFB$_1$)is a secondary metabolite produced by fungi of the genus Aspergillus (*Aspergillus flavus* and *A. parasiticus*) on oil based plant products including groundnuts and soybean. AFB$_1$ have been reported to be carcinogenic, mutagenic and immunotoxicto several animal species [12] [13]. Furthermore, the toxicity of this mycotoxin is reported not only to be dependent on the concentration of the ingested mycotoxin, but also on the age and species of the exposed animal, as well as on the duration of dietary exposure[12] [13] [14].

The toxicity of AFB$_1$ has been reported in several fish species including Indian major carp (*Labeorohita*) [15] [16]; Nile Tilapia (*Oreochromis niloticus*) [17]; Channel catfish (*Ictalurus puntatus*) [18]; Rainbow trout (*Onchorynchus mykiss*) [19]; Seabass (*Dicentrarchus labrax*) [20] and in Shrimps (*Peanus monodons*) [21]. However, there is a paucity of report on the effects of dietary AFB$_1$in clariid catfishes. In this study, we investigated the effects of dietary exposure to AFB$_1$on the innate immune response and growth performance of juvenile *Heterobranchus longifilis* catfish and attempt to determine the maximum tolerable limits of juvenile *H. longifilis* to dietary AFB$_1$ using the innate immune response and growth performance as biomarkers of tolerance.

Materials and Methods

Research grade AFB$_1$ used for the study were purchased from Sigma Aldrich (St Louis, M.O USA). Purity of this mycotoxin was ascertained to be greater than 98% before use. Other chemicals and reagents used for the study were purchased commercially at the highest degree of purity available.

Preparation of the basal diets

The basal diet was formulated according to [22], with slight adjustments, using the following ingredients (fish meal 19%, Soybean cake 37%, maize 32.25%, palm oil 1.0%, fish oil 6.0%, Starch binder 2.0%, vitamin/mineral premix 0.5%, Bone meal 1.0%, salt 0.25%) to meet the nutritional requirements of juvenile catfishes[8]. Formulated diets were then subjected to proximate analysis.

Preparation of the experimental AFB$_1$diets.

The AFB$_1$ diets were produced by adding 1 mg crystalline AFB$_1$ (Sigma Chemicals St Loius USA) to 1 mL chloroform (to produce 1 mg: 1,000 µL aliquot of AFB$_1$). The quantity of the solution required to produce the chosen concentrations of the AFB$_1$ in the experimental diets were then pipetted using an automated adjustable pipette into 100 mL volumetric flasks. This volume was then made up to the 100 mL mark with methanol. The ingredients for the basal diets were weighed, completely mixed and added to the liquid mixture of AFB$_1$ producing the following diets: Diet A (control 0.0 µg AFB$_1$.kg^{-1}); Diet B (5.0 µg AFB$_1$.kg^{-1}); Diet C (15.0 µg AFB$_1$.kg^{-1}); Diet D **(**45.0 µg AFB$_1$.kg^{-1}) and Diet E (90.0 µg AFB$_1$.kg^{-1}). The mixtures were subsequently blended and placed in a hot air oven for the methanol to evaporate. These were then pelletized with an extruder pelletizer after the addition of weighted portions of starch binders. The AFB$_1$ content of the compounded diets were then ascertained using the multi-mycotoxin LC-MS/MS method [10] and thereafter, individually packed in air

Adeyemo B. T. et al / J. Vet Biomed. Sci.

45

tight poly ethylene bags and stored in a deep freezer (at 2 - 4 ^{0}C) until use.

Experimental Design

450 juvenile *H. longifilis* catfish used for this study were procured from the fish hatchery, University of Agriculture, Makurdi and transported to the Aquaculture Research Unit of the Veterinary Teaching and Research Farm, Univerity of Abuja. The fishes were allowed to acclimatize to laboratory conditions for 21 days before the commencement of the experiment.

The fish culture experimental setup is as described [23]. Briefly, fifteen(15) 1000 L capacity tanks retrofitted with water inflow and out flow devices that were divided into five groups (each consisting of a triplicate set). The experimental fish were randomly allotted into different groups and fed any of diets A, B, C, D or diet E for 56 days. Using a hand held net, 5 fish were randomly sampled from each tank for immunological determinations at set time points day 7, 14, 28 and day 56. The sampled fish were weighed, length measured and then bled via caudal veni-puncture using a 23 G needle fitted on a 5 ml syringe. Aspirated blood was then emptied into 5 ml thin-bore glass test tubes.

Determination of Innate Immune Response

The innate immune response was evaluated by an assay of the lysozyme (mucus and serum lysozyme) activities; mucus alkaline phosphatase activity and the phagocytes respiratory burst activity.

Lysozyme activity

For the analysis of lysozyme concentrations, previously collected blood (without anticoagulants) were allowed to clot for 30 minutes and then centrifuged (3000 G for 5 minutes) after which clear serum was eluted using a 2 ml syringe. Before the lysozyme assays, collected serum were preheated in a water bath (at 56 ^{0}C for 30 minutes) to knock off the activity of complement system proteins and ensure the lysis of *Micrococcus lysodiekticus* occurred exclusively due to the action of serum lysozyme [24].

a) Serum Lysozyme Activity

Serum lysozyme activity (SLA) was estimated by the turbidimetric method as described by [25]. Briefly, using egg white lysozyme (1 mglysozyme mL^{-1}) as standard, and a suspension of *M. lysodeikticus* (0.2 mg.mL^{-1} 0.05 M sodium phosphate buffer $_p$H 6.2); Lysozyme working solution (1 ng. µL^{-1}) was obtained by diluting the stock solution a hundred folds with sodium phosphate buffer (NaH$_2$PO$_4$; 0.05 M; $_p$H 6.2) and a calibration curve developed. To produce the calibration curve, varying volumes (150; 175; 200; 250 and 275 µL) of the working solution were placed in clean 2 mL glass tubes and sodium phosphate buffer were added to complete the contents of each tube to 300 µL; and to each of the sample test tubes were added 300 µL of the serum samples. All Tubes (both the samples and the standards) were incubated at 35 ^{0}C for 2 minutes after which 300 µL of *M. lysodiekticus* suspension (0.2 mg. mL^{-1}) was added to complete the volume of the contents of each tube to 600 µL. A blank sample was prepared by adding 600µL sodium phosphate buffer to a clean test tube. Difference between the initial and final turbidity (Optical Density [OD] reduction) was measured between 0.5 and 10 minutes at 450 µm. Results were expressed using the OD reduction for each sample and the linear regression equation of lysozyme calibration curves used to determine serum lysozyme levels (µg. ml^{-1}).

b) Mucus Lysozyme Levels

Epidermal mucus was collected non-lethally from individual fish following sub-lethal anaesthetization (placing fish in 5 mg.L^{-1} clove oil till loss of righting reflex). The mucus collection was performed using the method of [26]. Briefly, individual fish was transferred into a poly-ethylene bag containing 10 ml 50 mM sodium chloride solution. The bags were gently shaken by hand for two minutes to initiate mucus exudation from the fish. The resulting fluids were collected from the bag and immediately centrifuged (1500 revolution per minutes for 10 minutes at 4^{0}C), the supernatants were eluted for lysozyme assay after preheating in a water bath at 56 ^{0}C for 30 minutes (as described for serum lysozyme).

Adeyemo B. T. et al / J. Vet Biomed. Sci.

46

Mucus Alkaline Phosphatase Activity

Mucus alkalinephosphatase activity was determined using the phenolphthalein monophosphate method [27] with Quimica clinica aplicada(QCA, Amposta Taragona Espana) alkaline phosphatase diagnostic kits following manufacturer's instructions. Briefly, deionized water (1 ml) was placed into clean and appropriately labelled test tubes and one drop of chromogenic substrate was added. These were mixed and incubated for 5 minutes at 37^0C to activate the enzyme. After activation, 0.1 ml of serum sample was added, mixed and incubated for 20 minutes at 37 ^{o}C. After that, 5 mL of ALP colour developer was added. The ALP colour developer was prepared by mixing one vial of colour developer salt to 250 ml of deionized water. The standard was prepared alongside with the serum sample by adding 1 mL of deionized water into a clean test tube and a drop of chromogenic substrate to activate the enzyme. After activation, 0.1 ml of standard was added, properly mixed and incubated for 20 minutes. Following incubation, 5 ml of ALP colour developer solution was added. The absorbance of the standard was read against a deionized water blank at 540 nm using a spectrophotometer (Spectrum Lab750, Sweden). Alkaline phosphatase concentration was obtained using the formula

$$\frac{\text{Absorbance of sample} \times 30}{\text{Absorbance of standard}} = \text{IU.L}^{-1}\text{of alkaline phosphatase}$$

Phagocytes Respiratory Burst Activity

Phagocytes respiratory burst activity (PRBA)was determined in peripheral blood using the Nitro Blue Tetrazolium (NBT) assay following the methods of [28]. Briefly, the wells of a 'U'-bottomed microtiter plates were filled with 50 µL blood and incubated for 1 hour at 35 ^{0}C (for cells to adhere). The supernatant non adherent cells were removed after a three times washing of the wells with buffered phosphate saline ($_p$H 7.4). 50 µL of 0.2 % NBT solution was then added and the plate incubated for another 1 hour. Cells in the wells were later fixed using absolute methanol (100% methanol) for 3 minutes, washed three times with 30 % methanol and air-dried. The optical density were read in an ELISA reader at 620 µm after 60 µL 2N potassium hydroxide and 70 µL dimethyl sulphoxide were added to dissolve the formazan precipitate formed.

Determination of Growth Performance

The effects of dietary AFB_1 on growth performance was determined by the evaluation of the Specific Growth Rate (SGR), percentage gain in total body length and percentage gain in body weight in a 56 days feeding period. The specific growth rate was determined as described by [29] using the formulae SGR = $100 \times [(\text{InW}_2 - \text{InW}_1)/t_2\text{-}t_1]$. Were, W_2 is weight attained after period of feeding on diet; W_1 is weight at commencement of the feeding experiment; $t_2\text{-}t_1$ is the time period (in days) of feeding on the experimental diets. Total body length was determined by the use of a ruler. Percentage gain in total body length was determined mathematically using the formula

$$\frac{\text{Final length (cm)} - \text{initial length (cm) x 100}}{\text{Initial length (cm)}}$$

Body weight was determined by the use of a sensitive Mettler scale (Mettler Toledo, Germany).The mean weight gain was determined by subtracting the weight at the start of the experiment from the weight at the end of the experiment.

The percentage gain in body weight was determined mathematically by the formula

$$\frac{\text{Final weight (g)} - \text{initial weight (g) x 100}}{\text{Initial weight (g)}}$$

Water samples were collected at the beginning of the experiments and at every sampling time points for the determination of the water quality parameters as described by [30].

Statistical Evaluation

Data analysis was carried out using a one-way analysis of variance (ANOVA), after the data has been assessed for normality and homogeneity (using the Kolmogorov-Smirnow test) and if necessary, appropriately transformed. The Duncan multiple range test was performed to separate variant means. All statistical evaluations were performed at the significant level of 5 % using SPSS (IBM, Version 20) software. Data are presented as

Adeyemo B. T. et al / J. Vet Biomed. Sci.

47

mean ± standard deviation of the mean of each culture tank (n = 30).

RESULTS

The AFB_1 content, nutrient composition and proximate analysis of the experimental diets are as presented in Table 1. It shows the concentrations of AFB_1 in the diets to be higher than the concentrations of the purified AFB_1 added to the various diets at the time of formulation. Hence, the concentration of the AFB_1 in the various diets would hereafter be appropriately quantified as follows, Diet A (2.0 $\mu g\, AFB_1\, kg^{-1}$); Diet B (7.3 $\mu g\, AFB_1\, kg^{-1}$); Diet C (17.6 $\mu g\, AFB_1\, kg^{-1}$); Diet D (48.0 $\mu g\, AFB_1\, kg^{-1}$) and Diet E (93.0 $\mu g\, AFB_1\, kg^{-1}$). Furthermore, there were no variations in the crude protein contents, metabolizable energy and the digestible energy levels of the produced diets (Table 1).

The results of the innate immune response of *H. longifilis* following dietary exposure to graded concentrations of AFB_1 are as depicted in Fig. 1, 2, 3, 4 and Figure 5.

Serum Lysozyme Activity

Figure (1) shows serum lysozyme activity (SLA) of *H. longifilis* juvenile catfish following dietary AFB_1. The SLA decreased with increasing concentrations of dietary AFB_1. At 7 days of feeding, the mean SLA of fish fed diet A was not significantly different (p > 0.05) compared with the mean SLA of fish fed diet B; it however, was significantly (p < 0.05) higher compared to the mean SLA of fish fed diets C, D and diet E.

At 14 days of feeding, one way analysis of variance (ANOVA) shows whereas the mean SLA of fish fed diet A was significantly (p < 0.05) higher compared to the SLA of fish fed diets amended with AFB_1, post hoc analysis reveals there were no variations in the mean SLA of fish fed diet D and fish fed diet E (p > 0.05).

At 28 days of feeding, one way ANOVA shows the mean SLA of fish fed diet A was not significantly different from the SLA of fish fed B (p > 0.05) but varied significantly from the SLA of fish fed diets C, D and diet E (p < 0.05).

At 56 days of feeding, one way ANOVA shows there were no variations in the mean SLA of fish fed diet A and diet B (p > 0.05), at this same period of sampling, post hoc evaluation shows the mean SLA of fish fed diet C was not significantly different (p > 0.05) from the mean SLA of fish fed diet D but differed significantly (p < 0.05) from the mean SLA of fish fed diet E (Fig. 1).

Epidermal Mucus Lysozyme Activity

The epidermal mucus lysozyme activity (EMLA) of fish fed dietary AFB_1 are as depicted in Figure (2). It shows dietary exposure to varying concentrations of AFB_1 caused a reduction in the EMLA of *H. longifilis*. Fig. 2 also shows the EMLA of fish fed diet A was not significantly different from the EMLA of fish fed diet B at 7 days of feeding (p > 0.05), they were however significantly different from the mean EMLA of fish fed diets C, D and diets E (p < 0.05).

At 14 days of feeding, the mean EMLA of fish fed diet A differed significantly from the mean EMLA of fish fed diets amended with varying concentrations of AFB_1. Further, post hoc analysis reveals the mean EMLA of fish fed diets amended with AFB_1 differed significantly from one another (p < 0.05).

At day 28 and day 56 of feeding, one way analysis of variance (ANOVA) shows there was no variation between the mean EMLA of fish fed diet A and diet B (p > 0.05). Post hoc evaluation shows the mean EMLA of fish fed diet A varied significantly (p < 0.05) from the mean EMLA of fish fed diets C, D and diet E (Fig. 2).

Epidermal Mucus Alkaline Phosphatase Activity

The mucus ALP activity of fish fed diets amended with AFB_1 were significantly lower compared with those of fish fed the control diet (Fig.3). Post hoc analysis further reveals there were no variations in the mucus ALP activity of fish fed diet B and diet C throughout the duration of the feeding experiment. Figure (3)

Adeyemo B. T. et al / J. Vet Biomed. Sci.

48

also show that in fish fed diet D and diet E, the mucus ALP activity varied significantly ($p < 0.05$) at Day 28 compared with Day 56 of feeding.

Phagocytes Respiratory Burst Activity
The phagocytes respiratory burst activity (PRBA) of juvenile *H. longifilis* catfish fed diets amended with AFB_1 are presented in Figure (4). At 7 days after commencement of the feeding, one way ANOVA shows the mean PRBA of fish fed diet A (the control diet) differed significantly ($p < 0.05$) from the mean PRBA of fish fed diets amended with varying concentrations of AFB_1; Post hoc analysis further reveals there is no variation between the mean PRBA of fish fed diet D and fish fed diet E ($p > 0.05$).

At day 14 post commencement of feeding, one way ANOVA shows there is no variation ($p > 0.05$) in the mean PRBA of fish fed diets A, D and diet E; Post hoc evaluation further shows whereas the mean PRBA of fish fed diet B was not significantly different from the mean PRBA of fish fed diet E ($p > 0.05$); and the mean PRBA of fish fed diet D and diet E do not vary significantly ($p > 0.05$), the mean PBRA of fish fed diet B varies significantly ($p < 0.05$) from the mean PBRA of fish fed diet D; and the mean PRBA of fish fed diet C also varies significantly from the mean PRBA of fish fed diet D.

At day 28, one way ANOVA shows the mean PRBA of fish fed diet A was not significantly different ($p > 0.05$) from the mean PRBA of fish fed diets B, D and diet E. Post hoc assessments further shows the mean PRBA of fish fed diets B, C and diet D varied significantly ($p < 0.05$) from one another.

Figure (4) also shows, that at day 56 of the feeding trial, the mean PRBA of fish fed diet A was significantly different from the mean PRBA of fish fed diets C, D and diet E ($p < 0.05$), post hoc test reveals the mean PRBA of fish fed diets C, D and E do not vary significantly ($p > 0.04$) from one another.

Growth Performance
The result obtained for the dietary exposure of aflatoxin B1 on growth response of *H. longifilis* are presented in Table 2 and figure 5.

Dietary AFB_1 caused significant reductions in the growth performance indices of *H. longifilis*. These changes were marked by reductions in the percentage gain in body length, reductions in percentage gain in body weight and reductions in the specific growth rate (figure 5). Post hoc test reveals fish fed diets amended with varying concentrations of AFB_1 (i.e. fishes fed diets B, C, D and Diet E), exhibited varying response in the assayed growth parameters. Furthermore, dietary AFB_1 elicited a diminution of growth along the gradients of concentration of AFB_1 and duration of dietary exposure to AFB_1 with the mathematically determined intercept of the exponential loss in weight and exponential loss in length being at about 20% and occurring at a dietary concentration of $AFB_1 \geq 17.6 \, \mu g. \, kg^{-1} \leq 48.0 \, \mu g. \, kg^{-1}$ (Fig. 5).

Table (2), shows the variation in the percentage gain in body length of fish fed Diet B and fish fed Diet C were not significantly different ($p > 0.05$); also, there were no significant variations in the percentage gain in body length of fish fed Diet D compared with those of fish fed Diet E. Also, the percentage gain in body length of fish fed Diet B and Diet C were significantly different from those of fishes fed Diet D and Diet E.

Analysis of the percentage mean weight gain of fishes fed diets amended with AFB_1 shows the variations in the mean weight gain of fish fed Diets B, C and Diet D not to be significantly different ($p > 0.05$). These however, varied significantly ($p < 0.05$) from the percentage mean weight gain of fish fed diet E (Tab. 2).

Table (2) further, shows there were no significant variations ($p > 0.05$) in the specific growth rate of fish fed the diets amended with AFB_1.

The results obtained for water quality assessments were within the recommended

Adeyemo B. T. et al / J. Vet Biomed. Sci.

49

ranges specified for catfish culture and are as depicted in table 3.

DISCUSSION

The results obtained for dietary concentrations of AFB_1 after the feed composition/production shows that AFB_1 contents of the various formulated diets were higher than the amounts of the purified AFB_1 added at the time of the diets production; these differences reflects the level of contamination of the feed ingredients as previously reported [23].

The water quality parameters were within the ranges specified for catfish culture and may thus not have contributed to the results obtained in the study [30].

No mortalities were observed in this study, contrary to the findings in juvenile hybrid sturgeon(*Acipenser ruthenus* and *A.baeri*) exposed to dietary AFB_1[31]. The reason for this may be that the concentrations of AFB_1 used for this study was not lethal to juvenile *H. longifilis* catfish and or that Sturgeons may be more sensitive to AFB_1 compared to *H. longifilis*[32].

Serum lysozyme activity has been suggested as a veritable indicator of innate immune response in teleost [33]. According to [34], serum lysozyme cleaves the β-1,4-glycosidic linkages between the N-acetyl muramic acid and N-acetyl glucosamine in the cell walls of Gram-positive bacteria thus preventing the invasion of Gram-positive bacteria. In addition, lysozyme is known to be opsonic in nature as they have the capacity to activate the complement system as well as phagocytes in teleost, hence aiding in the extenuation of the pathogenicity of some Gram negative bacterial pathogens.

Lysozyme is an enzyme that has been reported to be widely distributed throughout the body in fish and most animal species. It has been detected in serum, mucus, mucus membranes and in tissues rich in leukocytes. Lysozyme is reported to be mainly produced by monocytes/macrophages the neutrophils and in granules of eosinophilic granular cells of the

intestine [35]. In the present study, samples used for the assessments of lysozyme (i.e. serum and mucus samples) were preheated to 56 ^{0}C (for 30 minutes), hence the results obtained reflects only the lysozyme activities as the effects of complements and other bioactive moieties would have been inactivated by the heating process[36] [37] [38].

Dietary AFB_1 at an inclusion concentration greater than17.60 µg. kg^{-1}AFB_1induced a depression of the lysozyme (serum and epidermal mucus lysozymes) activities that were dependent both on the dietary concentration of $AFB_1$and on the duration of feeding. AFB_1 is known to be hepatotoxic, and this effect in fish generates a reduction in the serum total protein concentration resulting from the inhibition of protein synthesis following the binding of AFB_1adducts to cellular macromolecules [39] [40].

From the results of the present study, it appears the lysozyme response in *H. Longifilis* may be induced very rapidly and may not be unrelated to other alarm situations such as stress imposed on the fish by the dietary AFB_1. Thus lysozyme concentrations in *H. longifilis* may be in the overall, an alarm response acting as an acute-phase protein as previously noted [41]. Similar observations have been reported albeit in other animal species [42] [43]. Other probable mechanisms of these effects have been previously enunciated,wherein AFB_1 initiates haematopoetic toxicities (in both the proximal kidney and in the spleen) that elicits lymphocytolysis and a reduction in the immunoglobulin production [15] [16].

Alkaline phosphatase is a lysosomal enzyme reported to play a protective role in the fish during the early stages of wound healing [44]. The presence of alkaline phosphatase in the bodily fluids of fish and their roles in the non-specific defence mechanism of these organisms have also been described [25].

In the present study, the ALP activity of fish fed diets amended with AFB_1decreased throughout the duration of the feeding experiment. In

Adeyemo B. T. et al / J. Vet Biomed. Sci.

50

regards to the dietary concentration of AFB_1, The ALP activity of fish fed diet B was not significantly different from the ALP activity of fish fed diet C indicating, feed containing 7.3μg AFB_1 kg^{-1} elicited a similar ALP activity as feed containing 17.6μg AFB_1 kg^{-1} in juvenile *H. longifilis*; further, there were no variations in the ALP activity of fish fed diets amended with 48.0 μg AFB_1 kg^{-1} and 93.0 μg AFB_1 kg^{-1} at days 28 and 56 of the feeding experiment, suggesting the thresh hold for dietary AFB_1 Alkaline phosphatase activity in *H. longifilis* may be 17.6μg $AFB_1 kg^{-1}$.

Phagocytosis is one of the most important innate immune processes in poikilothermic animals, as it is the least influenced by the ambient temperature [45] [46]. The phagocytes (neutrophils and macrophages) eliminates phagocytised bacteria mainly by the production of reactive oxygen species during a so called 'respiratory burst activity' [47] [48].

The phagocytes respiratory burst activity is the rapid release of reactive oxygen species (superoxide anion and hydrogen peroxide) from immunocompetent cells to degrade internalized foreign particles (majorly bacteria). In these reactions, the immunocompetent cells utilize nicotinamide adenine dinucleotide phosphate to reduce O^{2-} to an oxygen free radical and later hydrogen peroxide [45].

This study shows in juvenile *H. longifilis* catfish fed diets amended with AFB_1, the phagocytes respiratory burst activity responded in a nonspecific way. For example, whereas there were no variations in the phagocytes respiratory burst activity of fish fed diet amended with 7.3 μg $AFB_1 kg^{-1}$ compared with those of fish fed the control diet (2.0 μg $AFB_1 kg^{-1}$), there were significant increases in the phagocytes respiratory burst activity of fish fed diets amended with AFB_1 at inclusion concentrations greater than or equal to 17.6μg $AFB_1 kg^{-1}$ at days 7, 14 and day 28 of the feeding experiment. This was then followed by a progressive reduction in the phagocytes respiratory burst activity (that culminated in fish fed diets amended with AFB_1 greater than or equal to 17.6μg $AFB_1 kg^{-1}$ having a phagocytes respiratory bust activity being lower than those of fish fed the control diet [2.0 μg $AFB_1 kg^{-1}$] at 56 days post commencement of the feeding trial).

This trend appear to imply, dietary AFB_1 at an inclusion rate greater than or equal to 17.6μg $AFB_1 kg^{-1}$ diets elevated the phagocyte respiratory burst activity in the first 28 days of exposure and subsequently caused a reduction in the phagocytes respiratory bust thereafter. The phagocytes respiratory bust activity is an energy dependent process, and the reduction in this parameter at day 56 seems to suggest exhaustion or a reduction of the biosynthesis of the essential protein molecules required for the process [32].

Also, dietary AFB_1 elicited a progressive leukocytopenia that was dependent on the dietary concentration of AFB_1 and on the duration of feeding. Dietary AFB_1 has been reported to elicit a depression of the peripheral leucocytes counts in a number of animal species [49] [13] [15].

Dietary exposure to AFB_1 elicited significant reductions in the percentage gain in body length, percentage gain in body weight and a reduction in the specific growth rate of juvenile *H. longifilis* catfish similar to the observations in Channel catfish (*Ictalurus punctatus)* [42], Nile tilapia (*Oreochromis niloticus*) [49] [50] and in Shrimps (*Peanusmonodons*) [51].

Whereas, the variations in the percentage gain in length and percentage gain in weight were not significant in fish fed diets amended with 7.3 μg AFB_1 kg^{-1} and 17.6 μg AFB_1 kg^{-1}, fish fed diets amended with 48.0 μg AFB_1 kg^{-1} and 93.0 μg AFB_1 kg^{-1} varied significantly only in terms of percentage gain in weight.

Concerning the dietary concentration of AFB_1, fish fed diet containing 17.6 μg AFB_1 kg^{-1} exhibited the best growth performance indices relative to fish fed the control diet. Hence, 17.6 μg AFB1 kg^{-1} diet may be the highest tolerable limits for AFB_1 in juvenile *Heterobranchus longifilis* catfish.

Adeyemo B. T. et al / J. Vet Biomed. Sci.

51

In summary, the results of the present study reveals the outcome of protracted dietary exposures to AFB_1 (even at minute concentrations of 17.6μg AFB_1 kg^{-1}dietin *Heterobranchus longifilis* catfish)is the suppression of the innate immune response marked by a reduction of the activity of lysozymes, reduction in the activity of mucus alkaline phosphates, and a reduction in the phagocytes respiratory burst activity. These changes are also accompanied by significant reductions in the growth performance indices of this important aquaculture fish species.

Acknowledgements

Conflict of interest: The authors declare no conflict of interest.

Author Contribution:

Conceptualization, A.B.T and E.G.N; Methodology, A.B.T., T.B, E.G.N., Formal analysis, E.A., Writing (Original draft), A.B.T, and E.A., Reviews and Editing, E.G.N and E.A

REFERENCES

1.   Food and Agriculture Organization of the United Nations (FAO) (2004). Worldwide regulation of mycotoxins in food and feed in 2003. FAO Food and Nutrition paper No 81. Food and Agriculture Organization of the United Nations, ISBN 9251051623, Rome, Italy. pp. 1728-3264.

2.   Myhr Anne, Dalmo R (2005) Introduction of genetic engineering in aquaculture: Ecological and ethical implications for science and governance. Aquaculture 250: 542-554. 10.1016/j.aquaculture.2004.12.032.

3.   Food andAgricultural Organization (FAO) (2014) The state of the World Fisheries and Aquaculture: Opportunities and Challenges E-ISBN 978-82-5-108276-8 (PDF)
www.fao.org/publicaitons

4.   Naylor RL, Hardy RW, Bureau DP, Chiu A, Elliot M, Farrell AP, Forster I, Gatlin DM, Goldberg RJ, Hua K, Nicholas PD (2009) Feeding aquaculture in an era of finite resources. Proceeding of the National Academy of Sciences 106: 15103-15110.

5.   Spring P, Fegan DF (2005). Mycotoxins–a rising threat to aquaculture. Feedmix13:5- 9.

6.   Tacon AGJ, Hasan MR, Subasinghe RP (2006) Use of fishery resources as feed inputs for aquaculture development: trends and policy implications. FAO Fisheries Circular. Food and Agricultural Organization of the United Nations, Rome, p 99

7.   Gatlin DM, Barrows FT, Brown P, Dabrowski K, Gaylord TG, Hardy RW, Herman E, Hu GS, Krogdahl A, Nelson R, Overturf K, Rust M, Sealey W, Skonberg D, Souza EJ, StoneD, Wilson R, Wurtele E (2007) Expanding the utilization of sustainable plant products in aquafeeds: a review. *Aquac Res* 38: 551-579.

8.   Ayinla OA (2007) Analysis of feeds and fertilizers for sustainable aquaculture development in Nigeria. Ph.D thesis. Nigerian Institute of Oceanography and Marine Research. 215 pp.

9.   Bankole SA, Mabekoje OO (2004) Occurrence of aflatoxins and Fumonisins in pre-harvest maize from south-western Nigeria. Food Add Contam 21(3): 251-255.

10.   Monbaliu S, Van poucke C, Detavermier C, Dumoulin F, Van D Velde M, Schoeters E, Van Dyck S, Averkieva O, Van peteghem C, De Saeger S (2010) Occurrence of mycotoxins in feed as analyzed by a multi-mycotoxin LC-MS/Ms method. *J Agric Food Chem* 58: 66-71

11.   Santos GA, Rodrigues I, Naechrer K, Encarnacao P (2010) Mycotoxins in

Adeyemo B. T. et al / J. Vet Biomed. Sci.

52

Aquaculture: occurrence in feed components and impact on animal performance. *Aquacult Europe* 35: 6-10.

12. International Agency for the Regulation of Cancer IARC. (1993) Some naturally occurring substances in food items and constituents, heterocyclic aromatic amines, and mycotoxins. IARC monographs evaluation of carcinogenic risk in human, Vol 56, pp 489-521 International Agency for Research on Cancer World Health Organization, Lyon, France.

13. Hendricks JD (1994) Carcinogenicity of aflatoxins in non-mammalian organisms. In: Eaton DL, Groopman JD (eds) The toxicology of aflatoxins: human health, veterinary and agricultural significance. Academic Press, New York. pp 103-136.

14. Santacroce MP, Conversano M, Casalino E, Lai O, Zizzadoro C, Centoducati G, Crescenzo G (2008) Aflatoxins in aquatic species: Metabolism, toxicity and perspectives. *Rev Fish Biol Fish*. 18: 99-130.

15. Shoo PK, Mukherjee SC. (2001) Immuno suppressive effects of afflation B1 in Indian major carp (*Labeorohita*). Comp ImmunolMicrobiol Infect Dis 24: 143-149.

16. Shoo PK, Mukherjee SC, Jain AK, Mukherjee A (2003) Histopathological and electron microscopic studies of gill and opisthonephros of Rohu, *Labeorohita* to acute and subchronic aflatoxin B1 toxicity. Asian Fish Sci. 16: 257-268.

17. Anh T, Grizzle J, Lovell R, Manning B, Rottinghaus G (2002) Growth and hepatic lesions of Nile tilapia (*Oreochromisniloticus*) fed diets containing aflatoxin. *Aquaculture* 212 (1): 311-319.

18. Manning BB, Menghe HL, Robinson EH (2005) Aflatoxins from moldy corn cause no reductions in channel catfish *Ictaluruspunctatus* performance. *J of World AquacultSoc* 36 (1): 59-67.

19. Williams DE, Orner G, Willard KD, Tilton S, Hendricks JD, Pereira C, Benningh off AD, Bailey GS (2009) Rainbow Trout (*Oncorhynchusmykiss*) and ultra-low dose cancer studies. *Comp Biochem Physiol C: ToxicolPharmacol* 149 (2): 175-181.

20. El-sayed YS, Khalid RH (2009) Toxicity biochemical effects and residue of aflatoxin B1 in marine water-reared sea bass (*Dicentrarchuslabrax L.*). *Food and Chem. Toxicol.* 7: 1606-1609.

21. Radhika G, Rajain PR, Kizhakkayil CG, Nandiath KS (2012) Ultrastructural changes in hepatopancreas of *Peneausmonodon* Fabricius given aflatoxin B1 diet. *Aquacult Res* 43: 32-43.

22. Adeyemo BT, Tiamiyu LO, Ayuba VO, Cheikyula JO (2016) Effects of dietary fumonisin B1 on haematology and growth performance of the clariid fish *Heterobranchus longifilis. J Agric Vet Sci* 9 (8): 26-33.

23. Adeyemo BT, Tiamiyu LO, Ayuba VC, Musa S, Odo J (2018) Effects of dietary mixed aflatoxin B1 and fumonisin B1 on growth performance and haematology of juvenile *Clariasgariepinus* catfish. Aquaculture 491: 190-196. https: //doi.org /10.1016/*j.aquaculture.* 2018.03.026.

24. Subramanian S, MacKinnon SLM, Ross NW (2007) A comparative study on the innate immune parameters in the epidermal mucus of various fish species. *Comp Biochem Physiol* 148: 256-263.

25. Ellis AE (1990) Lysozyme assays. In: Stolen JS, Fletcher TC, Anderson DP, Robertson BS, Muiswinkel W.B (eds), Techniques in Fish Immunology. SOS publications, USA p. 101-103.

26. Ross NW, Firth KJ, Wang A, Burka JF, Johnson SC (2000) Changes in hydrolytic enzyme activity of naïve Atlantic salmon *Salmosalar* skin mucus due to

Adeyemo B. T. et al / J. Vet Biomed. Sci.

53

infection with s a l m o n l o u s e *Lepeoptheriussalmonis* and cortisol implantation. *Dis. Aqua Or*g 41: 43-   51.

27.   Mullivnor RA, Hanning VL, Harris H (1985) Quantitative analysis of alkaline phosphatase in serum and amniotic fluid: comparison of biochemical and immunologic assays. *J Lab Clin Med* 105: 342-348.

28.   Secombes CJ (1990) Isolation of salmonid macrophages and analysis of their killing activity. In: Stolen JS, Fletcher DP, Anderson DP, Robertson BS, Muiswinkel VWB (eds.) Techniques in fish immunology. SOS Publications, New Jersey. pp 137-154.

29.   Arnason T, Bjornson B, Steinarsson A, Oddgeirsson M (2009). Effects of temperature and body weights on growth rate and feed c o n v e r s i o n r a t i o i n T u r b o t (*Scohthalmusmaximus*). *Aquaculture* 295: 218-225.

30.   Boyd CE, Tucker CS (1992) Water quality and pond soil analysis for aquaculture. Agricultural experiment station, Auburn University, Auburn. Alabama, USA. pp. 183.

31.   Rajeev RP, Zhu X, Lei W, Han D, Yang Y, Xie S (2011) Low Levels of Aflatoxin B1 could cause mortalities in juvenile hybrid s t u r g e o n, *A c i p e n s e r r u t h e n u s* m a l e *A. baeri* female. *Aquacult. Nut*. 17(2):39-47.

32.   Manning BB (2005) Mycotoxins in aquaculture. In: Diaz D.E (ed) The mycotoxin blue book Context, United Kingdom. p. 139-156.

33.   Paulsen SM, Lunde H, Engstad RE, Robertsen B (2003) In vivo effects of β-glucan and LPS on regulation of lysozyme activity and mRNA expression in Atlantic s a l m o n (*Salmosalar*L.). *Fish Shellfish Immunol* 14 (1): 39-54.

34.   Tort L, Balasch JC, Makenzie S (2003) Fish immune system. A crossroads between innate and adaptive responses. *Immunología* 22 (3): 277-286.

35.   Sveinbjornsson B, Olsen R, Paulsen S (1996) Immunocytochemical localization of lysozyme in intestinal eosinophilic granule c e l l s (E G C s) o f A t l a n t i c s a l m o n, *Salmo salar* L. *J Fish Dis*. 19: 349-355.

36.   Lie O, Evensen O, Sorensen A, Froysadale E (1989) Study of lysozyme activity insome fish species. *Dis Aqua Org* 6 (1-5): 1-5.

37.   Cuesta A, Ortuno J, Rodriguez A, Esteban MA, Meseguer J (2002) Changes in some innate defence parameters of seabream (*Sparusaurata*L.) induced by retinol acetate. *Fish and Shellfish Immunol* 13 (4): 279-291.

38.   B a l f r y S K, I w a m a G K (2 0 0 4) Observations on the inherent variability of measuring lysozyme activity in coho salmon (*Oncorhynchuskisutch*). *Com Biochem Physiol B* 138 (3): 207-211.

39.   Macé K, Aguilar F, Wang JS, Vautravers P, Gómez-Lechón M, Gonzalez FJ, Groopman J,   Harris CC, Pfeifer AM (1997) Aflatoxin B1-induced DNA adduct formation and p53 mutations in CYP450-expressing human liver cell lines. *Carcinogenesis* 18(7): 1291-1297.

40.   S a n t a c r o c e M P, N a r r a c c i M, Acquaviva MI, Cavallo RA, Zacchino V, Centoducati G (2011) New development in aflatoxin research: from aquafeed to marine cells. In: Irineo Torres-Pacheco I (ed.) Aflatoxins-Detection, Measurement and Control. InTech, Croatia 209-234.

41.   Demers NE, Bayne CJ (1997). The immediate effects of stress on hormones and plasma lysozyme in rainbow trout. *Develop Comp Immunol* 21(3): 63-73.

42.   Jantrarotai W, Lovell RT (1990) Subchronic toxicity of dietary aflatoxin B1 to channel catfish. J Aqua Ani Health 2: 248-254.

Adeyemo B. T. et al / J. Vet Biomed. Sci.

54

43. Qureshi MA, Brake J, Hamilton PB, Hagler WM, Jr. Nesheim S (1998) Dietary exposure of broilers to aflatoxin results in immune dysfunction in progeny chicks. *Poult Sci.* 77: 812-819.

44. Iger Y, Abraham M (1994) The process of skin healing in experimentally wounded carp. *J Fish Biol* 36: 421-437.

45. Magnadottir B (2006). Innate immunity of fish (overview). *Fish Shellfish Immunol* 20: 137-51.

46. Magnadottir B, Jonsdottir H, Helgason S, Bjornsson B, Jorgensen TO, Pilstrom L (2001) Immuneparameters of immunised cod (*Gadusmorhua*L). *Fish Shell fish Immunol* 10: 75-89.

48. Mary VS, Theumer MG, Arias SL, Rubinstein HR (2012) Reactive oxygen species sources and biomolecular oxidative damage induced by aflatoxin B1 and fumonisin B1 in rat spleen mononuclear cells. *Toxicol.* 302 (2):299-307.

49. Chávez-Sánchez MC, Martínez Palacios CA, Osorio Moreno I (1994) Pathological effects of feeding young *Oreochromisniloticus*diets supplemented with different levels of aflatoxin B1. *Aquaculture*127: 49-60.

50. Tuan NA, Grizzle JM, Lovell RT, Manning BB, Rottinghaus GE (2002) Growth and hepatic lesions of Nile tilapia (*Oreochromisniloticus*) fed diets containing aflatoxin B1. *Aquaculture* 212 (1): 311-319.

51. Boonyaratpalin M, Supamattaya K, Verakunpiriya V, Suprasert D (2001) Effects of aflatoxin B1 on growth performance, blood components, immune function and histopathological changes in black tiger shrimp (*Penaeusmonodon*Fabricius). *Aquac Res* 32: 388-398..
.

Adeyemo B. T. et al / J. Vet Biomed. Sci.

55

Table 1. **Ingredients, Proximate Composition and AFB$_1$ contents of Diets Amended With Aflatoxin B1**

	Aflatoxin B1 amended diets				
Ingredients	A	B	C	D	E
[#]Fish meal (%)	19.00	19.00	19.00	19.00	19.00
Soybean cake (%)	37.00	37.00	37.00	37.00	37.00
Maize (%)	32.25	32.23	32.23	32.25	32.25
Palm oil (%)	1.00	1.00	1.00	1.00	1.00
Fish oil (%)	6.00	6.00	6.00	6.00	6.00
Vit/min Premix (%)	0.50	0.50	0.50	0.50	0.50
Bone meal (%)	1.00	1.00	1.00	1.00	1.00
NaCl (%)	0.23	0.25	0.25	0.25	0.25
AFB$_1$	2.0	7.3	17.6	48.0	93.0
[$]Starch Binder (%)	2.00	2.00	2.00	2.00	2.00
Crude Protein (%)	40.01	40.00	40.04	40.01	40.02
Gross Energy (kj/g)	19.77	20.00	20.01	19.86	20.00
Digestible Energy (kj/g)	12.00	12.01	11.98	12.01	12.10
TotalLipids (%)	9.00	9.00	9.00	9.00	9.00
Moisture (%)	2.30	2.30	2.30	2.30	2.30
Ash (%)	9.72	9.70	9.7	9.69	9.58

A = 2.0 µg AFB$_1$kg^{-1}; B = 7.3 µg AFB$_1$kg^{-1}; C = 17.6 µg AFB$_1$kg^{-1}; D = 48.0 µg AFB$_1$kg^{-1}; E = 93.0 µg AFB$_1$kg^{-1}; # Fish meal of 72 % crude protein; $ cassava starch binder

Adeyemo B. T. et al / J. Vet Biomed. Sci.

56

Table 2: **Growth performance of *Heterobranchus longifilis* fed varied levels of AFB$_1$ for 56 days**

Parameter	A	B	C	D	E
Initial total length (cm)	28.07 ± 0.11^a	27.99 ± 0.61^a	28.01 ± 0.03^a	28.05 ± 0.30^a	28.03 ± 0.07^a
Final total length (cm)	45.11 ± 0.09^a	35.30 ± 0.10^b	34.90 ± 0.17^b	32.80 ± 0.17^b	32.84 ± 0.11^b
Gain in length (%)	60.71 ± 0.17^a	26.12 ± 0.40^b	24.60 ± 0.16^b	16.93 ± 0.01^c	17.16 ± 0.37^c
Initial weight (g)	146.3 ± 0.33^a	147.0 ± 1.09^a	148.0 ± 1.07^a	146.2 ± 1.32^a	147.0 ± 1.81^a
Final weight (g)	185.5 ± 1.14^a	169.9 ± 0.67^b	169.9 ± 0.63^b	168.3 ± 1.39^b	163.9 ± 1.17^c
Gain in weight (%)	39.15 ± 1.01^a	22.18 ± 0.89^b	21.84 ± 0.67^b	22.12 ± 0.41^b	16.96 ± 0.66^c
specific growth rate (SGR)	0.42 ± 0.01^a	0.25 ± 0.12^b	0.25 ± 0.05^b	0.25 ± 0.03^b	0.20 ± 0.03^b

Rows with different superscripts are different. A=diet 2.0 µg AFB$_1$ kg^{-1}; B= diet 7.3 µg AFB$_1$ kg^{-1}; C= diet 17.6 µg AFB$_1$ kg^{-1}; D= diet 48.0 µg AFB$_1$ kg^{-1}; E= diet 93.0 µg AFB$_1$ kg^{-1}.

Table 3 **Water quality parameters of *Heterobranchus longifilis* culture tanks following a 56 days feeding trial with graded concentration of AFB$_1$**

Parameter	Values obtained
Dissolved oxygen (mgL^{-1})	6.34 ± 1.07
Total alkalinity (mgL^{-1} CaCO$_3$)	49.58 ± 7.62
Nitrate-N (µg L^{-1})	403.5 ± 17.49
Nitrite-N (µg L^{-1})	39.76 ± 19.18
Ammonia- Nitrogen (µg L^{-1})	163.5 ± 79.03
$_pH$	$7.24 \pm 0,52$
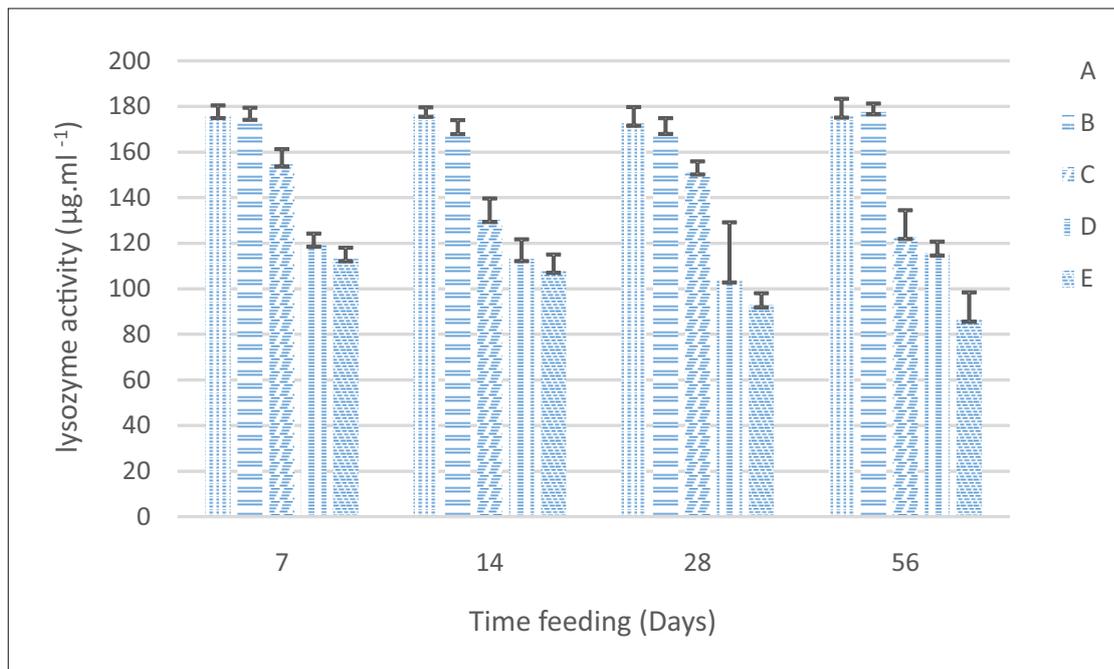
Adeyemo B. T. et al / J. Vet Biomed. Sci.

57

A=diet 2.0 μg AFB_1 kg^{-1}; B= diet 7.3 μg AFB_1 kg^{-1}; C= diet 17.6 μg AFB_1 kg^{-1}; D= diet 48.0 μg AFB_1 kg^{-1}; E= diet 93.0 μg AFB_1 kg^{-1}.

Figure 1. Serum lysozyme activity of juvenile *Heterobranchus longifilis* catfish fed diets amended with aflatoxin B1.
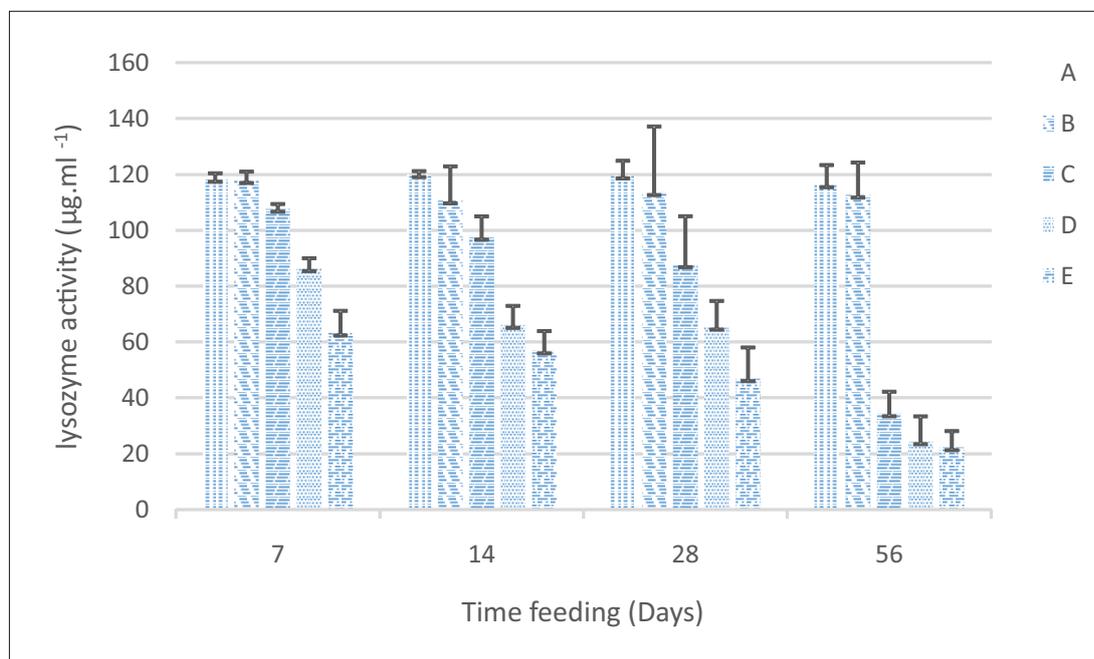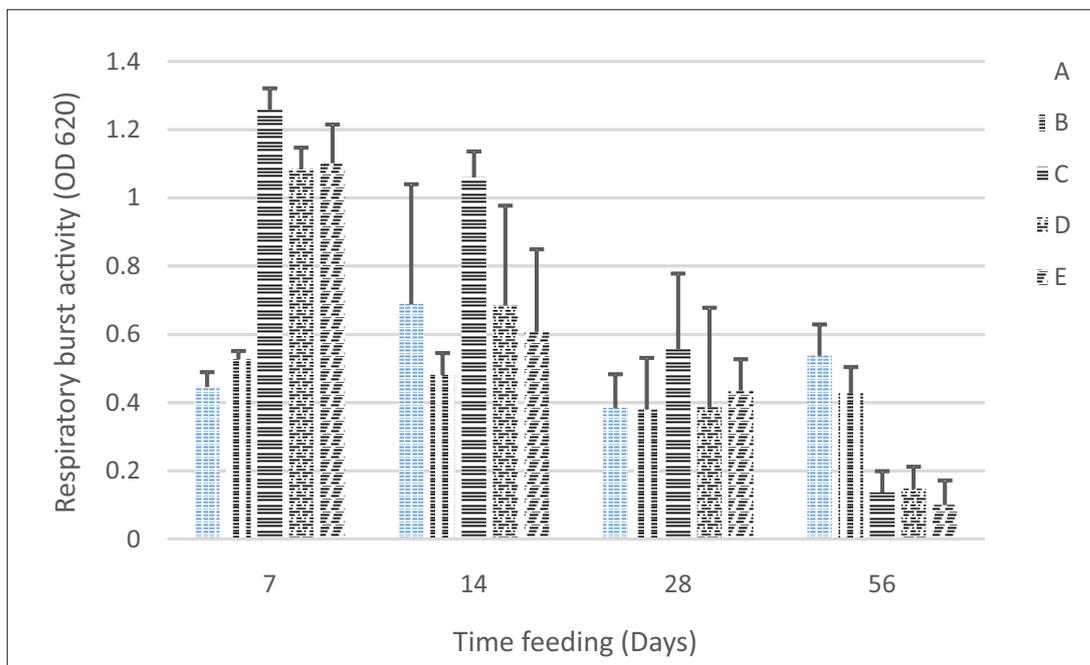

A=diet 2.0 μg AFB_1 kg^{-1}; B= diet 7.3 μg AFB_1 kg^{-1}; C= diet 17.6 μg AFB_1 kg^{-1}; D= diet 48.0 μg AFB_1 kg^{-1}; E= diet 93.0 μg AFB_1 kg^{-1}.

Figure 2. Epidermal mucus lysozyme activity of juvenile *Heterobranchus longifilis* catfish fed diets amended with aflatoxin B1.

Adeyemo B. T. et al / J. Vet Biomed. Sci.

58
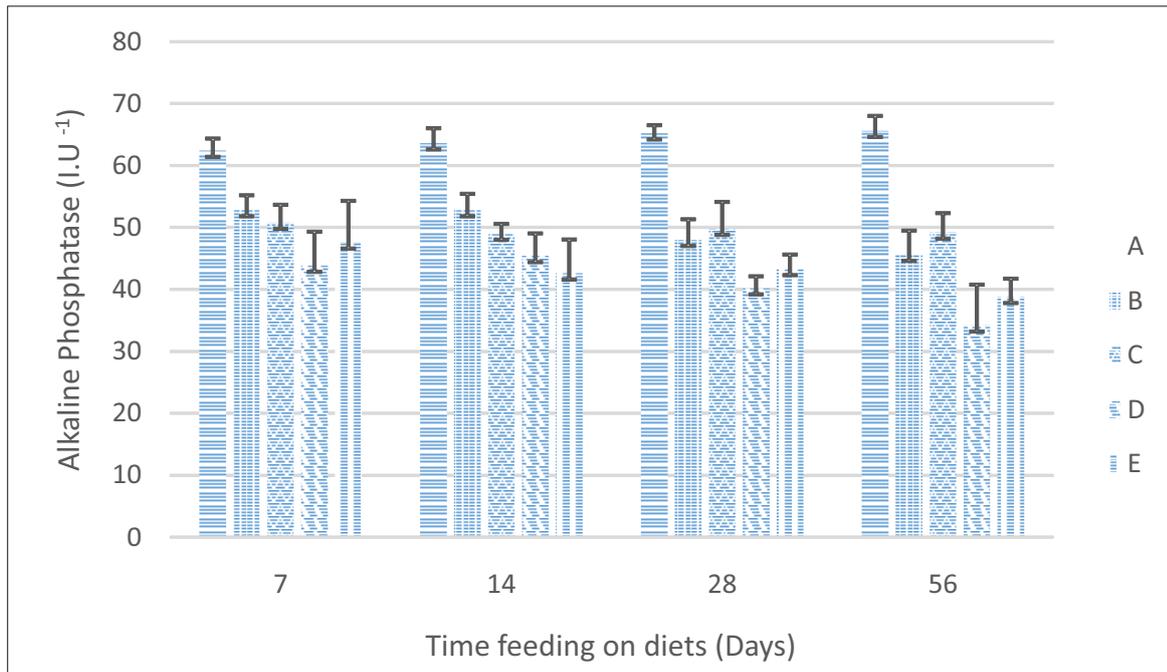
A=diet 2.0 µg AFB_1 kg^{-1}; B= diet 7.3 µg AFB_1 kg^{-1}; C= diet 17.6 µg AFB_1 kg^{-1}; D= diet 48.0 µg AFB_1 kg^{-1}; E= diet 93.0 µg AFB_1 kg^{-1}.

Figure 3. Epidermal mucus alkaline phosphatase activity of juvenile *Heterobranchus longifilis* catfish fed diets amended with aflatoxin B1.

A=diet 2.0 µg AFB_1 kg^{-1}; B= diet 7.3 µg AFB_1 kg^{-1}; C= diet 17.6 µg AFB_1 kg^{-1}; D= diet 48.0 µg AFB_1 kg^{-1}; E= diet 93.0 µg AFB_1 kg^{-1}.

Figure 4. Phagocytes respiratory burst activity of juvenile *Heterobranchus longifilis* catfish fed diets amended with aflatoxin B1.

Adeyemo B. T. et al / J. Vet Biomed. Sci.

59
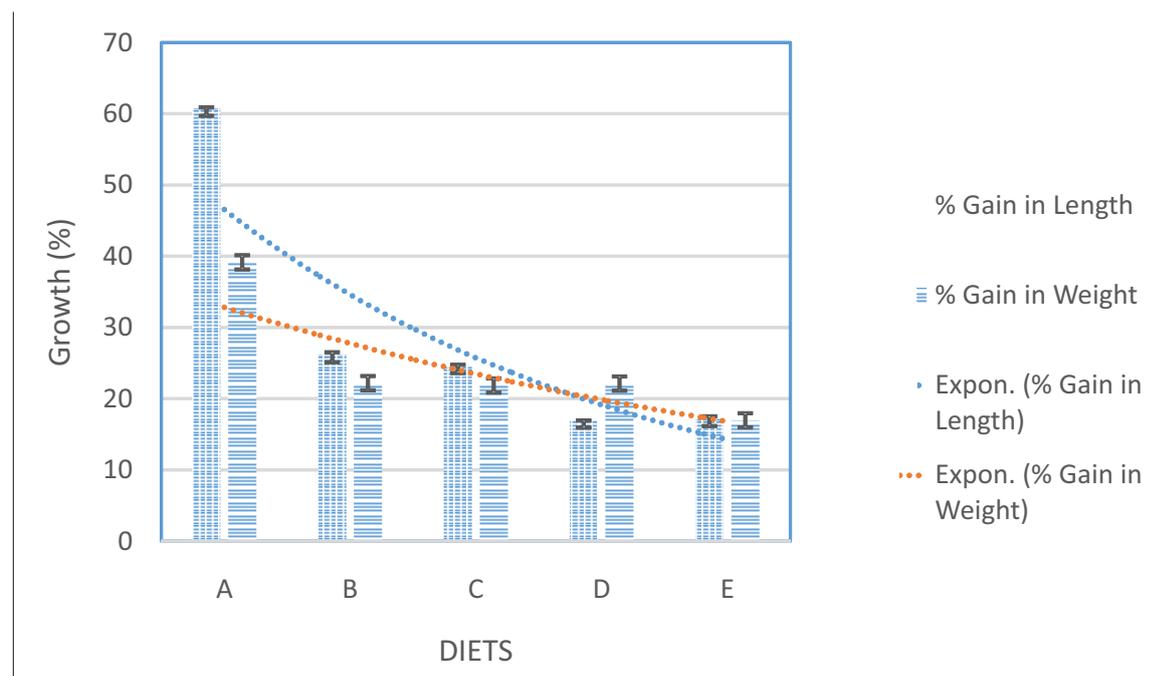
A=diet 2.0 µg AFB_1 kg^{-1}; B= diet 7.3 µg AFB_1 kg^{-1}; C= diet 17.6 µg AFB_1 kg^{-1}; D= diet 48.0 µg AFB_1 kg^{-1}; E= diet 93.0 µg AFB_1 kg^{-1}.

Fig. 5   Length-weight relationship of juvenile *Heterobranchus longifilis* catfish fed diets amended with aflatoxin B1.