

Full Paper

Status of Expression of GnRH gene in Fed, Starved, and Refed *Clarias Gariepinus* (African Catfish)

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Abstract

Inadequate nutritional status impairs gonadal activities, which may induce reproductive difficulties in *Clarias gariepinus*. This research was aimed at evaluating the influence of starvation and subsequent refeeding on the expression of GnRH gene in *Clarias gariepinus*. One hundred and eighty (180) healthy juvenile *Clarias gariepinus* (African catfish) used in this study were randomly placed into two groups in triplicates. Fish in the control group were fed to satiation while fish in the treatment group were starved and refed and the fish in the control group were continuously fed. Brain tissue samples were collected after 7 days and 14 days of starvation, histopathology, and gene expression studies were done. The histological sections of brain tissue of week one starved and fed, *C. gariepinus* show neuronal cells on a background of neuropil while week two starved reveal badly distorted morphological architecture and degenerated neuronal cells. However, the week two re-fed, *C. gariepinus* brain tissue shows vacuolation of neuropil while the week two continuous fed shows no abnormalities. No mortality was recorded during starvation and the entire experimental period. In the second week of starvation, a decrease in the fold change (1.7) was observed when compared to week one while the fold change (1.2) observed in week three (week one of re-fed) is lower than the fold change observed in week one and two of starvation. The fold change (1.7) observed in week four (week two re-fed) is higher than the fold change observed in week three. The highest fold change (2.13) was observed in week one of starvation while the lowest fold change (1.24) was observed in week one of re-feeding. This study reveals that exposure of *C. gariepinus* to prolonged food deprivation alters the expression of GnRH, which might lead to impairment in the reproductive success of *Clarias gariepinus*.

Keywords: *Clarias gariepinus*, gene expression, GnRH, histopathology, starvation

Introduction

Recent years have witnessed interest in African catfish, *Clarias gariepinus*, for fish farming not only in African countries but also in European countries. The possibility for high stocking densities, food conversion, resistance to disease, low requirements for quality water, and excellent meat quality are the main factors promoting African catfish.[2].

Fish is a vital source of high-quality protein providing approximately 16% of the animal protein consumed by the world's population [8]. It is an important protein source in regions where livestock is relatively scarce. Fish culture is one of the fastest-growing sectors of the world's animal production, to sustain a such high rate of increase in production, reproductive success is vital for an aquaculture system.

Reproduction is a biological process that results in the production of a new individual. In fish, the nervous and endocrine (neuroendocrine) work together to control reproduction. The neuroendocrine mechanism regulates reproduction through the hypothalamus-pituitary-gonadal (HPG) axis. The hypothalamus of the brain is the major site responsible for the production of the neuropeptide gonadotropin-releasing hormone (GnRH), which is the primary factor controlling reproduction and other important hormones which include gonadotropin-inhibiting hormone (GnIH), kisspeptin and neurokinin [13]. GnRH is a critical component of the hypothalamus-pituitary gonadal (HPG) axis as it stimulates gonad; the release of gonadotropins (follicle stimulating hormone (FSH) and luteinizing hormone (LH) by pituitary gonadotropes cells [16, 7] gonadotropins then stimulate gonad development and the release of sex steroids leading to gametogenesis. GnRH has been identified in several fish, including African catfish for which two GnRH variants have been isolated: cfGnRH or GnRH1 and GnRH2 [16, 1].

In fish, as in mammals, feeding and reproduction are linked processes [9] as successful reproduction requires adequate resources in order to sustain high energy demand for the production of gametes.

Several research studies have reported on the effects of starvation and subsequent re-feeding which focus on muscle growth, metabolic responses, hematological indices, and biochemical parameters on Nile tilapia [5] However, most of the research studies were conducted to identify the effect of starvation on reproductive hormone in *Clarias gariepinus*. Hence this research study aims to determine the influence of starvation and re-feeding on GnRH gene expression in *Clarias gariepinus*. This study will provide the basis for improved guidelines in farming, thereby helping to optimize the production of *Clarias gariepinus* in aquaculture.

Materials and Methods

Experimental Animals

A sample of 180 healthy juvenile *Clarias gariepinus* (African catfish) was obtained from a fish farm at Lakwe Ajah, Lagos. The fish were kept in fiber tanks to acclimatize for two weeks and were fed three times daily to satiation with a commercial feed. After acclimatization, the tanks were assigned in triplicates for two groups thus: the control group (feeding group) and the experimental group (starving group) for 14 days and re-feeding for 14 days. i.e., 3 tanks each were used for each group. Thirty (30) fish were stocked in each triplicate fiber tank. The experiment was carried out in two phases. In phase 1, fish in the control group were continuously fed to satiation with commercial feed for 28 days. The experimental group was starved for 14 days. In phase 2, fish in the experimental group (starving group) were now re-fed together with the control group three times daily to satiation at 9 a.m., 2 p.m., and 6 p.m. using the same commercial feed for another 14 days. Water quality was maintained by renewing the water twice weekly.

RNA/ Histopathology, Sample Collection

After acclimatization, fish samples were collected before they were divided into two groups (feeding and starving). Subsequent samples were collected from each group after the 7th and 14th day (feeding and starving groups) and the 21st and 28th (continuous feeding and refeeding) days of experimentation. The brain was dissected to collect the brain tissue and immediately immersed in RNALater in 2 ml Eppendorf tubes and in 10% Formaldehyde for gene expression and histopathological studies respectively.

Total RNA Extraction/Quantification

The brain tissues were homogenized in an Eppendorf tube in the presence of lysis buffer (BME added) using a hand-operated plastic pestle to get through to the cell nucleus. The lysate was treated and DNase-free RNA was obtained. The purity was determined using a Thermo fisher 2000C spectrophotometer at 260/280 nm wavelength.

cDNA Synthesis/ Quantification

Complementary DNA (cDNA) was synthesized using reverse transcriptase polymerase chain reaction (RT-PCR). The synthesized cDNA was quantified using the primers shown in table 1 the synthesized cDNA was quantified using housekeeping β -actin (housekeeping, primers shown in table 1).

Table 1: Primers used for Nucleic acid quantification and qPCR.

S/n	Primer	Forward	Reverse
1	β -actin	TGGCCGTGACCTGACTGAC	CCTGCTCAAAGTCAAGAGCGAC
2	GnRH	GGATGGTGGTGTGTGTTGTG	CTGGTACTCTGGTCACGACTC

Quantitative PCR (qPCR)

Quantitative PCR was performed using 5x Hot Fire Pol Evergreen qPCR Supermix, prepared according to the manufacturer protocol. The qPCR Profile steps for β -actin and GnRH were carried out using the PCR profile: Initial denaturation at 95 °C for 15 sec (1 cycle), 40 cycles of denaturation at 95 °C for 15 sec, annealing 57 °C for 1 min, elongation 72 °C for 30 sec and final elongation at 72 °C for 30 sec.

Statistical Analysis

All data resulting from the experiment were analyzed by one-way variance (ANOVA) using the SPSS (Statistical Package Computer, Software 2015 version, Chicago Illinois, USA). Duncan's multiple range test for the least significant difference was deployed to compare differences among individual means. Differences were considered at $p < 0.05$ (Zar, 1999). Results are presented in means for each group.

Results

The histological sections of brain tissue of week one starved and fed, *C. gariepinus* are shown in Figures 1 and 2. The result shows neuronal cells on a background of neuropil with no abnormalities seen. Histological sections of two weeks starved, and two weeks fed are shown in Figures 3 and 4, respectively. These plates reveal badly distorted morphological architecture and degenerated neuronal cells.

Week one re-fed *C. gariepinus* histological section is shown in Figure 6 with normal brain tissue. However, the week two re-fed, *C. gariepinus* brain tissue shows vacuolation of neuropil as shown in Figure 5 while week one and week two continuously fed as shown in Figures 7 and 8 shows no abnormalities. The mean weight (50.91±0.62 g) of week One and (51.5±0.71 g) of week two re-fed *C. gariepinus* did not show any significantly different from the starved ($p > 0.05$). The mean weight of week one starved fish was observed to be significantly lower than the initial weights (60.02±0.57 g) at the commencement of the experiment ($p < 0.05$). The mean weight (50.2±0.91 g) of week two starved fish was also significantly lower ($p < 0.05$) than

the initial mean weight (54.07±0.73 g) of week Two starved and the initial weight (60.07±0.35 g) as shown in Table 2. In the fed group, an exponential increase in weight was observed in week two, which is significantly higher when compared to the control. The maximum increase in weight was obtained after week four of feeding though not statistically different from week three of feeding. After week four of feeding, the weight (106.91±1.2 g) of *C. gariepinus* was impacted being significantly higher than the initial weight (60.02±0.57 g), ($p < 0.05$). Although, there was no significant difference in the weight between weeks one fed and the initial weight of the fish. No mortality was recorded during starvation and the entire experimental period.

Table 3: shows the extracted RNA concentration ng/μl and $\Delta 260/280$ value of fed, starved, and re-fed *Clarias gariepinus*. This revealed the RNA concentration of week one and two starved *C. gariepinus* as 18.7 ng/μl and 16.32 ng/μl, and purity 1.95 and 1.90 respectively, 15.52 ng/μl and 20.77 ng/μl concentration and purity 1.91 and 1.91 was observed in week one and week two of re-fed *C. gariepinus*. In the control group, the concentration observed from week one to week four is shown.

The mean gene expression of fed, starved, and re-fed *Clarias gariepinus* revealed the fold change in gene expression between and within groups as shown in table 4. In the second week of starvation, a decrease in the fold change (1.7) was observed when compared to week One while the fold change (1.2) observed in week three (week one of re-fed) is lower than the fold change observed in weeks one and two of starvation. The fold change (1.7) observed in week four (week two re-fed) is higher than the fold change observed in week three. The highest fold change (2.13) was observed in week one of starvation while the lowest fold change (1.24) was observed in week one of re-feeding. one week starved and re-fed *C. gariepinus* did not show any significant difference in the gene expression. However, there were significant differences in the expression of GnRH gene in two weeks of starved and re-fed *C. gariepinus*. Figure 9: shows the mean gene expression level of starved and re-fed *C. gariepinus*.

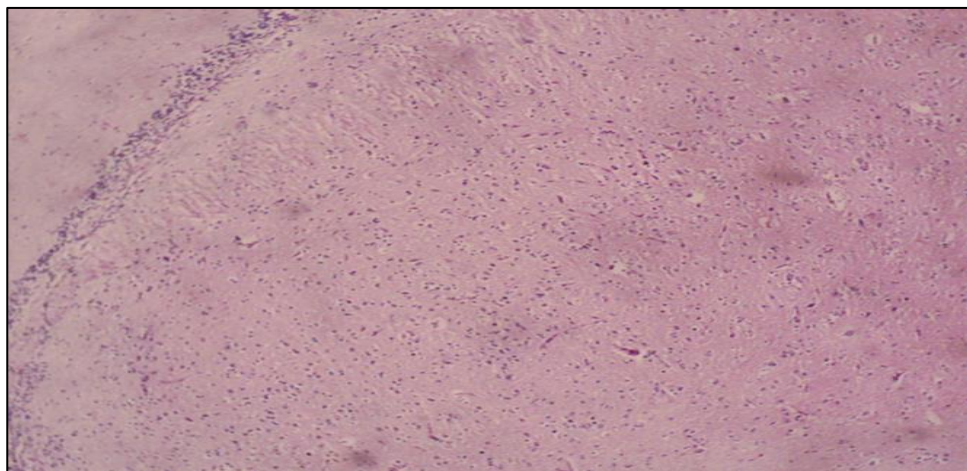


Figure 1: Plate 1: Histological section of brain tissue of week one starved *Clarias gariepinus*. (X100 H & E STAIN)

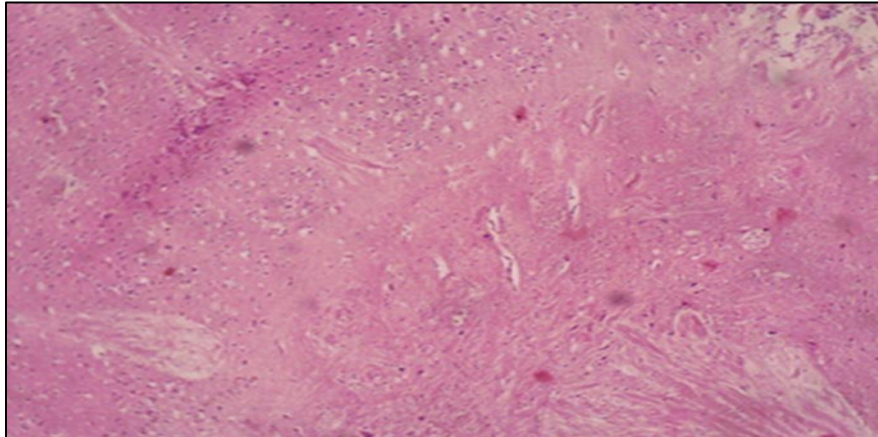


Figure 2: Plate 2: Histological section of brain tissue of week one fed *Clarias gariepinus*. (X100 H & E STAIN)

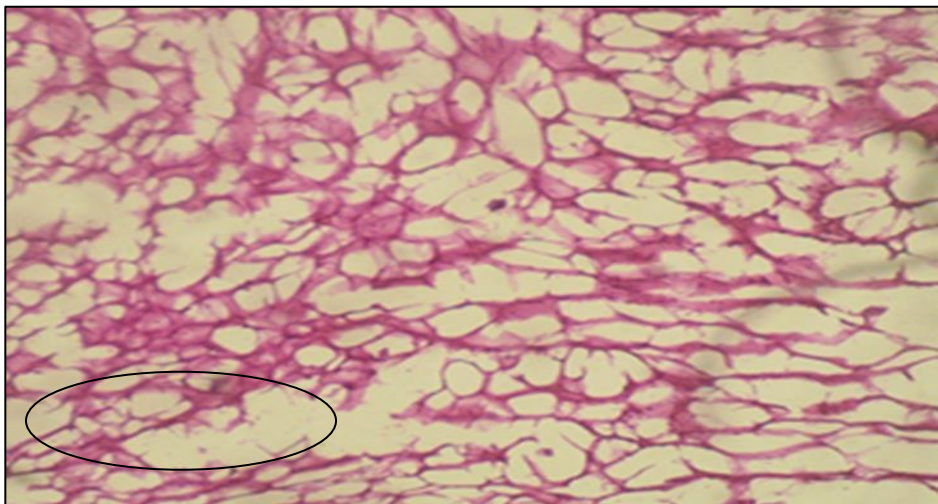


Figure 3: Plate 3: Histological section of brain tissue of week two starved *Clarias gariepinus*. (X100 H & E STAIN)

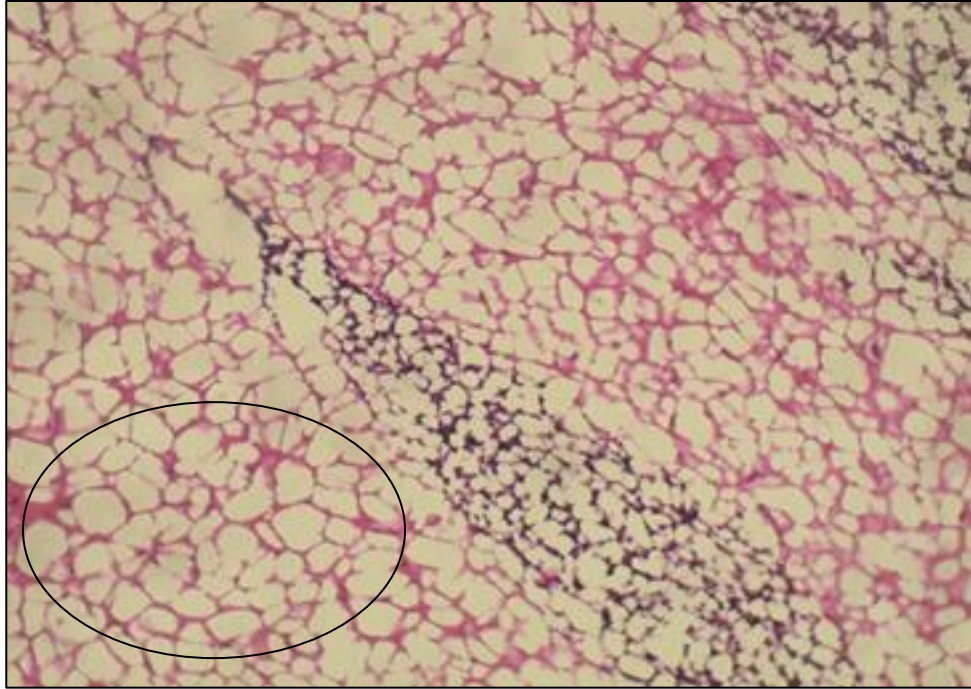


Figure 4: Plate 4: Histological section of brain tissue of week two fed *Clarias gariepinus*. (X100 H & E STAIN)

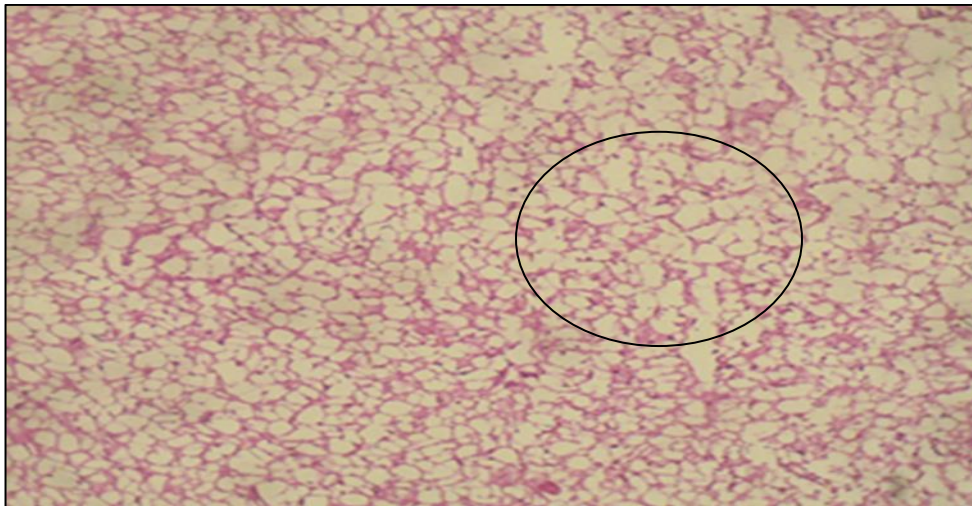


Figure 5: Plate 5: Histological section of brain tissue of week two re-fed *Clarias gariepinus*. (X100 H & E STAIN)

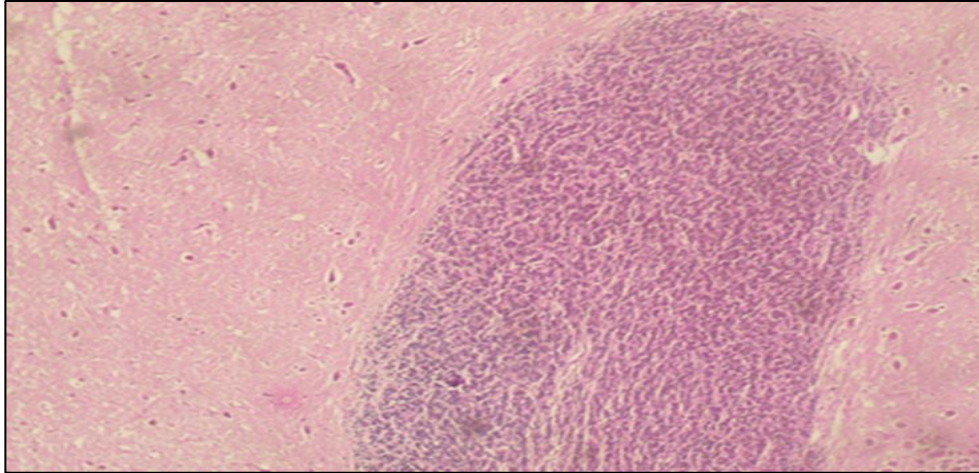


Figure 6: Plate 6: Histological section of brain tissue of week one refed *Clarias gariepinus*. (X100 H & E STAIN)

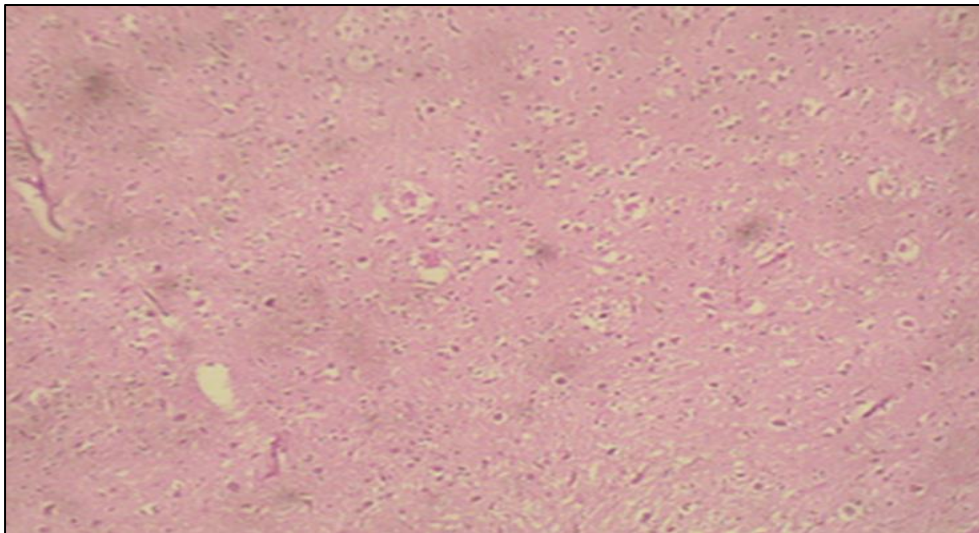


Figure 7: Plate 7: Histological section of brain tissue of week one continuous fed *Clarias gariepinus*. (X100 H & E STAIN)

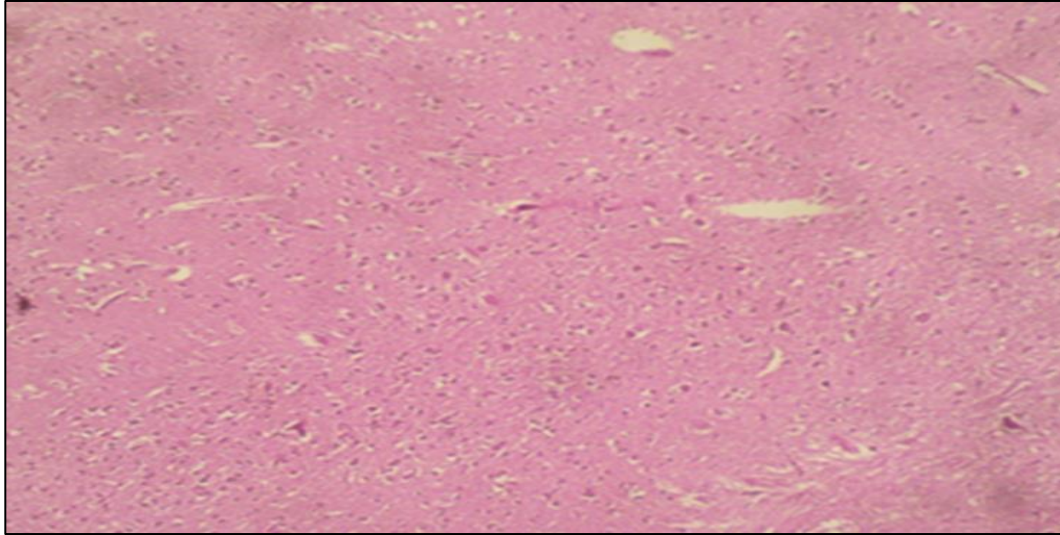


Figure 8: Plate 8: Histological section of brain tissue of week two continuously fed *C.gariepinus* (X100 H & E STAIN)

Table 2: Mean Weight Response of *Clarias gariepinus*

Weeks	W0	W1	W2	W3	W4
Fed Group	60.02 ± 0.57 ^a	64.57 ± 1.3 ^a	82.75 ± 0.44 ^b	97.97 ± 3.7 ^c	106.91 ± 1.2 ^c
STVD/Re-Fed	60.07 ± 0.35 ^c	54.07 ± 0.73 ^b	50.2 ± 0.91 ^a	50.91 ± 0.62 ^a	51.5 ± 0.71 ^a

Mean values in each row with similar superscripts are not significantly different (p>0.05).

- Key:** STVD= starved
 W0 = Week Zero
 W1 = Week One
 W2 = Week Two
 W3 = Week Three
 W4 = Week Four

Table 3: Nucleic Acid quantification showing the $\Delta 260/280$ values and concentrations in ng/ μ l of starved/fed and re-fed/continuous feeding *Clarias gariepinus*

Sample	Week	Concentration (μ l)	Purity ($\Delta 260/280$)
Treatment (starved)	One	18.7	1.9525
Control (fed)		18.45	2.1275
Treatment (starved)	Two	16.325	1.9025
Control (fed)		20.35	2.0025
Treatment (re-fed)	Three	15.525	1.915
Control (fed)		19.35	1.905
Treatment (re-fed)	Four	20.775	1.91
Control (fed)		18.975	1.9175

Values are means of four replicates

Table 4: The mean gene expression level of fed, starved and re-fed *Clarias gariepinus*

Sample	Week	Bactin ct	GnRH ct	Δ ct	$\Delta\Delta$ ct	Fold change
Control	1	25.65375	18.19	-7.46375	0	1
Treatment		27.09	18.5325	-8.5575	-1.09375	2.134280801
	2					
Treatment		26.385	18.145	-8.24	-0.77625	1.712673327
	3					
Treatment		26.205	18.4225	-7.7825	-0.31875	1.247249421
	4					
Treatment		26.4225	18.1475	-8.275	-0.81125	1.754731143
	5					

Values are in means of four replicates by each week

Key: β actin=Reference gene
 GnRH=Gene of interest
 Ct =Cycle threshold

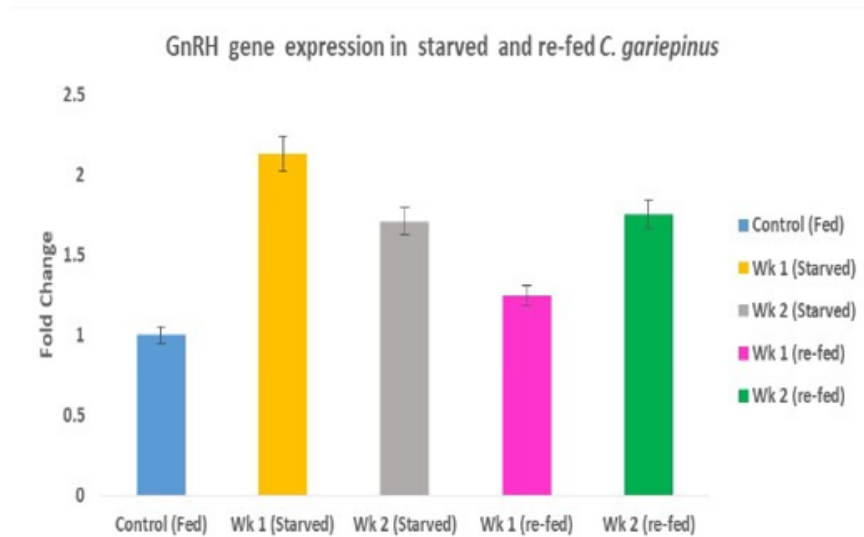


Figure 9: Bar chart indicating GnRH gene expression level of starved and re-fed *Clarias gariepinus*. Value for e each week are presented in mean

Discussion

In this study, we investigated how nutritional restriction and subsequent re-feeding can influence reproduction in *Clarias gariepinus*, change in body weight was also monitored. The reduction in body weight that was observed in week one and week two of starvation is an indication that food deprivation or starvation can cause weight loss in *C. gariepinus*. Similarly, this finding was observed in *Oreochromis niloticus*, Linnaeus [5]. Food deprivation causes the degradation of endogenous sources of energy (lipid, glycogen, and proteins) in order to maintain the fish's physiological homeostasis, leading to weight loss [15]. After re-feeding, an increase in weight was observed but the initial weight was not regained as observed in Persian sturgeon (*Acipenser Persicus*) [11] and in hybrid tilapia (*O. mossambicus* and *O. niloticus*) [10], where initial weight was regained after re-feeding. The continuous increase in body weight observed in the fed group with significant differences between weeks one and two signifies the importance of food and the impact it has on fish body weight; it could also mean that the feed used in feeding the fish is of good nutritious quality. The highest body weight gain which was observed in week four of feeding indicates the nutritional status of the fish as continuous growth as expected. Weight changes in the starved group, significantly differ from the initial weight.

The current study, the histology results of two weeks of starved *C. gariepinus* brain tissue showed badly distorted morphological architecture and degenerated neuronal cells. This result suggests that starvation may have caused an alteration in the cells of the brain, also reported desquamation of the apical part of mucosal of intestinal epithelium and liver showing a collapsed cytoplasm in starved *Cyprinus carpio* (17). During starvation, essential processes in fish are maintained on at the expense of accumulated (endogenous) energy reserves, resulting in the progressive depletion of tissue. This finding is similar in the liver of starved angel fish *Pterophyllum scalere* [3]. There were no observed abnormalities in the brain tissue of one week-starved *C. gariepinus*. In the fed group, no abnormalities were seen in week one of the starved fish. This result is also similar to the finding in fed *Cyprinus carpio* (17). However, in week two fed fish showed badly distorted and degenerated neuronal cells, these results appear to be dependent on some

experimental conditions which were not put into consideration during the cause of this study. During week one re-feeding, neuronal cells with no abnormalities were observed suggesting that the fish had recovered the distorted brain. The same trend was observed in the continuous feeding group. In the second week of re-feeding, neuronal cells were seen with no abnormalities whereas, abnormalities were seen in the continuous feeding group indicating that other conditions were responsible for the observed abnormalities in the brain tissue of fed *C. gariepinus*.

The concentration and purity of RNA extracted from the brain tissue sample of *C. gariepinus* suggest that it was good enough for the downstream application and this is in accordance with the previous work of RNA extracted from glass Catfish *Kryptopterus vitreolus* [9].

GnRH plays an important role in gonad maturation in vertebrates including catfish. This study showed that starvation significantly decreases GnRH expression in African catfish (*Clarias gariepinus*). This result is in accordance with those found in glass catfish (*kryptopterus vitreolus*) for which starvation caused a decrease in GnRH expression levels in the brain [4]. The same observation was in *Astatotilapia boturni*, a mouthbrooding African cichlid fish [14], *Oreochromis niloticus* [6]. The difference in the fold change observed between week one and week two starved fish clearly indicates the downregulation of GnRH gene as the starvation period is prolonged. This suggests that a longer starvation period would have more effect on gene expression and as such reproduction will be greatly impaired. However, the higher fold change seen in the second week of re-feeding when compared to week one re-feeding, shows that the gene was upregulated as a result of the food intake. In this study, one-week starvation did not show any significant effect on GnRH gene expression in the brain. This suggests that one-week food deprivation may not have an effect on the reproductive success of *C. gariepinus*.

Conclusion

This study suggests that exposure of *C. gariepinus* to prolonged food deprivation results in deleterious effects on the expression of the reproductive hormone, which might lead to impairment in the reproductive success of the commonest aquaculture specie *Clarias gariepinus*. This result may have possible implications in aquaculture practices as the production of quality gametes is exceptionally important to achieve reproductive success.

Conflicts of Interest

Authors must declare all relevant interests that could be perceived as conflicting. If no conflicts exist, the authors should state this.

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Supporting Information

The data sets used and/or analyzed during this study are available from the corresponding author upon reasonable request.

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