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Hepatic mRNA Expression of SOD and CAT activities and Antioxidant Profile in Blood of Juvenile African Catfish Exposed to Transport Stress

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Abstract

This study was conducted to evaluate stress responses of juveniles African catfish exposed to transportation for 6hrs duration in plastic bags. The fish were separated into two groups; control and treatment. The fish in the treatment group were placed in nylon bags with 7.5 liters of oxygenated freshwater in each bag. The bags were labeled according to the duration of transport as T1, T3, and T6 and the transportation was carried out in triplicate while the control group was not transported. Blood and liver samples were collected as follows; immediately after transportation, three days after transportation and six days after transportation for biochemical and molecular assay. In African catfish juveniles, transportation showed a substantial (P <0.05) increase in SOD and CAT mRNA expression shortly after transportation and a considerable (P <0.05) decrease on the third and sixth days after transportation. The result of the antioxidant profile of SOD, CAT, and GSH indicated significant increase (P <0.05) immediately after transportation, third and sixth day after transportation. It is concluded that live transportation process is a significant stressor as it results in significant changes in biochemical parameters and liver of African catfish juveniles.

Keywords: Catfish, Stress, Transport, Antioxidant, mRNA

INTRODUCTION

Live fish transport is an unavoidable operation in fish farms, however it causes stress, which can lead to decreased growth, greater mortality, and disease risk (Akinrotimi, 2010), physical injury (Crosby *et al*., 2005), and an increase in rearing expenses (Gomes *et al*., 2006). The

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general physiological reaction of an organism to threatening conditions and difficulties in the environment is referred to as stress. Stress that is frequent and protracted may have a deleterious impact on growth and development, immunity, and reproductive mechanisms. High - stress farming conditions can lead to decreased fish activity and well-being, putting fish welfare at risk (Segner *et al.,* 2012). It is critical to reduce stress levels during transportation in order to support survival rates and growth performance (Navarro *et al.,* 2016). This can be accomplished by adding chromium (Cr) (Rakhmawati *et al*., 2018), anesthetics and chemical additives (Velisek *et al.,* 2006), probiotics (Mohapatra *et al.,* 2013), non-iodized salt and palm oil (Idowu *et al.,* 2016), and oxygen(Orina *et al.,* 2014).

Reactive oxygen species (ROS) play a vital part in the immune system's function, as it helps to ensure a redox balance and activates multiple cellular signaling pathways. Excessive production of reactive oxygen species destroys cellular lipids, proteins, nucleic acids, membranes, and organelles, which can trigger apoptosis mechanisms (Redza-Dutordoir *et al*., 2016). Antioxidant defenses like superoxide dismutase (SOD), catalase (CAT), as well as reduced glutathione (GSH) help to prevent the harmful effects of reactive oxygen species (ROS). Such antioxidants protect biological components from oxidative damage caused by ROS. The imbalance between the formation and neutralization of ROS by such antioxidant systems within the organism is referred to as oxidative stress (OS), and it has become a popular issue in both terrestrial and marine toxicity research. The production of reactive oxygen species in aquatic animals triggers OS pathways in tissues, resulting in alterations in antioxidant enzyme activity (Romero *et al.,* 2007). As a result, antioxidant profiles were frequently used as OS biomarkers (Kumari *et al*., 2014).

Stress gene regulation and transcriptional expression in aquatic organisms are indications of physiological conditions (Shaheen *et al*., 2014). They give us the ability to understand the processes that respond to diverse stressors, as well as the ability to analyze stress reactions in varied situations (Eissa and Wang, 2016). The reaction of fish to stress involves a wide range of metabolic processes and pathways (Tort and Teles, 2012). which are linked to stress genes found in various organs and tissues, including the heart, liver, and muscles, which are expressed and quickly modified (Staib *et al.,* 2007).

The African catfish (*Clarias gariepinus*) is of enormous economic significance because it is the most abundant freshwater fish commonly produced and consumed in Nigeria (Ekasari *et al.,* 2019). In order to promote the health of *Clarias gariepinus* juveniles, it is necessary to research their stress reactions.The aim of this present study was to evaluate the effect of transportation on survival of African catfish juveniles, antioxidant profiles and hepatic mRNA expression of SOD and CAT activities.

MATERIALS AND METHODS

The Study Area

The acclimation and recuperation phase was carried out at the University of Lagos' Department of Marine Sciences, while the biochemical and molecular assay was done at the Nigerian Institute of Medical Research (NIMR) in Yaba, Lagos state. The fish were carried throughout the University of Lagos Akoka's campus in Lagos, Nigeria.

Weather Condition
The transportation was carried out from 11.00 am to 5.00 pm during the rainy season (September, 2021). Weather conditions on the day of transportation were as follows: no rainfall, cloudy and ambient temperature 26°C.

Source of Fish and Acclimatization
Clarias gariepinus juveniles were purchased from a commercial fish farm in Lagos state, Nigeria. Fish were stocked in concrete tanks filled with fresh water from the faucet and allowed to acclimatize for three days. During this time, the fish were fed a commercial floating feed twice a day (9:00 a.m. & 16:30 p.m.) until they were full. The feeding of the fish was halted 24 hours prior to the transfer process.

Fish Transportation
The fish were handled before being segmented into two groups: a control group (45 fish dispersed into three plastic tanks with a density of 15 fish per tank) that was not transported (stationed at the aquaculture unit), and a transport group (containing 360 fish distributed into nylon bags with a density of 40 fish per bag) which was subjected to transportation stress at 6hrs duration for one day. Therefore the total of 405 fish was used for the study and the procedures were carried out in triplicate. The transparent nylon bags (18 by 32 inches) were first doubled to prevent leakage and 40 fish were counted into each bag filled with 7.5 liters of freshwater. Excess air was removed from the bags and replaced with oxygen from oxygen tank, approximately 75% of the volume in the bags contained oxygen. The mouth of the bags were twisted tightly and secured with rubber bands. T1, T3, and T6 were assigned to the bags based on their transport length. They were then loaded onto a vehicle (Honda Odessy). when the fish were returned, they were kept in labeled plastic tanks for recovery, and they were also done in triplicate to the same density as the control group.

Samples Collection for Biochemical Assay
The blood and liver samples for transported fish in plastic bags and the control group were taken immediately after transportation (day 0), at day 3 and day 6 to determine the effect of transportation stress on the fish.
Blood samples were also taken via the caudal vein and collected in sample bottles (lithium heparin bottles) using 2ml syringes at successive time intervals (immediately after transportation, day 3 and day 6) for the experimental groups in order to assay for the antioxidant parameters (superoxide dismutase, catalase, and reduced glutathione).

Samples Collection for Molecular Assay
Fish were dissected with sterilized surgical blade from the belly side and cutting a line not too deep all the way up to the gills. The fish were opened up for better observation of the organ and then the liver was taken out with a dissection forceps which was collected in sterilized sample bottles containing RNA-later(R) and preserved in the refrigerator at 4°C for molecular assay. Before every collection for different treatments, the dissection instruments were sterilized again in methylated spirit to ensure no contamination. The liver samples were collected immediately after transportation (day zero), day 3 and day 6 for both experimental groups. Samples were used to determine mRNA expression of SOD and CAT using β-actin as housekeeping gene.

Determination of Antioxidant Activities
CAT was assayed colorimetrically at 620nm and expressed as moles of hydrogen peroxide (H2O2) consumed/min/mg protein as described by Quinlan *et al.* (1994). The reaction

mixture (1.5ml) contained 1.0ml of 0.01M pH 7.0 phosphate buffer, 0.1ml of plasma and 0.4ml of 2M H_2O_2. The reaction was stopped by the addition of 2.0ml of dichromate-acetic acid reagent (5% potassium dichromate and glacial acetic acid were mixed in 1:3 ratio). Total SOD activity in tissue homogenates was assessed using a modified version of Marklund and Marklund's (1974) method. The method is based on the ability of SOD to inhibit the autoxidation of pyrogallol. In 970 µL of buffer (100mMTris-HCl, 1mM EDTA, pH 8.2), 10µL of homogenates and 20µL pyrogallol (13Mm) were mixed. Assay was performed in thermostated cuvettes at 25°C and changes of absorption were recorded by a spectrophotometer at 480nm. One unit of SOD activity was defined as the amount of enzyme can inhibit the auto-oxidation of 50% the total pyrogallol in the reaction. Reduced glutathione (GSH) level was determined by the method of Ellman, (1959). To the homogenate was added 10% TCA (equal volume) and centrifuged. 1.0 ml of supernatant was treated with 0.5 ml of Ellman's reagent (19.8 mg of 5, 5′-dithiobisnitro benzoic acid (DTNB) in 100 ml of 0.1% sodium nitrate) and 3.0 ml of phosphate buffer (0.2M, pH 8.0). The absorbance was read at 412 nm.

CAT mRNA Expression and Hepatic SOD Determination

Total Ribonucleic acid (RNA) Extraction

The samples were extracted using the NIMR Biotech Ribonucleic acid Extraction kit (Nigerian institute of medical research Yaba, Lagos Nigeria) according to the manufacturer's instructions. The sample was homogenized with 300ul of lysis buffer, followed by 200ul of lysis buffer after grinding, incubated at room temperature for 5 minutes, and centrifuged at 10,000 rpm for 10 minutes. The upper aqueous phase was collected into another tube and 300ul of isopropanol and vortex were added, after which 500ul of chloroform was added and centrifuged again. The mixture was placed in the spin column and centrifuged for 30 seconds at 10,000 to 12,000 rpm. The collection tube was dried on tissue paper after the flow-through was disposed. The flow-through was removed and the collection tube was dried on tissue paper after 700ul of the primary wash buffer was introduced to the spin column and spun at 10,000 to 12,000 rpm for 30 seconds. The flow-through was removed and the collecting tube was blotted on tissue paper after 700ul of secondary wash buffer was introduced to the spin column and spun at 10,000 to 12,000 rpm for 30 seconds. To remove all traces of ethanol, the spin column was centrifuged at 12,000 to 14,000rpm for 2 minutes. The spin column was then put into another microcentrifuge tube and 50ul elution buffer or nuclease-free water was added to the center of the column and incubated at room temperature for 2 minutes. To extract the RNA, it was centrifuged at 10,000rpm for 1 minute and kept at -80°C. The purity and concentration of the RNA samples were measured using a Nanodrop with a 1µL aliquot (Thermo Scientific Nano Drop ONE).

Complementary DNA (cDNA) Synthesis

Following the manufacturer's instructions, Complementary DNA was synthesized using Solis BiodyneFirescript RT cDNA synthesis Mix with Oligo (dT) kits (Solis Biodyne, Tartu Estonia) The reaction components were thawed and combined in a nuclease-free microcentrifuge tube at the following programmed temperatures: primer annealing at 25°C for 5 mins, reverse transcription at 55°C for 15 minutes, and enzyme inactivation at 85°C for 5 mins.

Quantitative Polymerase Chain Reaction Method

To determine the genetic expression of the SOD1 and CATH1 genes, a quantitative PCR reaction was used. According to the manufacturer's instructions, the qPCR was done in a BioRad CFX96 Deep Well Real-Time System using a 5x Hot FirepolEvaGreen® qPCRsupermix. It's a prepared combination that includes Firepol® DNA polymerase with a

chemical modification that allows for hot start, an optimized hot-start PCR buffer, MgCl2, dUTPs, dNTPs, and EvaGreen dye.

The reaction was carried out in a 20µl volume with 1µM EvagreenqPCRsupermix (5x), 1µM primers, and 2µl of cDNA. To make up the 20µl volumes, nuclease-free water was used. Initial denaturation was done at 95°C for 2 minutes, followed by 40 cycles of 95°C for 30 seconds and 60°C for 30 seconds. The melt curve was started at 55°C for 5 seconds and increased by 5°C every 5 seconds until it reached 95°C. To eliminate variability in cDNA amount and quality, the β -actin housekeeping gene was employed to normalize the data. The CFX program was used to evaluate the qPCR results, and the 2-ΔΔCT method was used to analyze the relative quantification of gene expression among the treatment groups (Livak and Schmittgen, 2001).

Table 1: Primers sequences used for the study

S/N	GENE	FORWARD	REVERSE
1	CAT	TCTGTTCCCGTCCTTCATCC	ATATCCGTCAGGCAATCCAC
3	BETA-ACTIN	GCCTCTCTGTCCACCTTCCA	GGGCCGGACTCATCGTACT
4	SOD	GCATGTAGGAGACTTGGGCAAT	CCGTGATTTCTATCTTGGCAACA

Data Analysis

To determine whether there were significant differences in antioxidant and molecular parameters between days, one-way analysis of variance was performed using SPSS version 23.0 and results were presented with means ±SD of three replicates. The Duncan multiple range test (DMRT), a post hoc test, was employed to investigate further significant changes across variables over days, with P ≤ 0.05 considered statistically significant.

RESULTS AND DISCUSION

Results

Effect of Transportation Stress on Fish Survival

Immediately after transportation, reduced activity was observed in fish transported in plastic bags. There was no mortality in the first few hours of transportation but with increase in days, mortality set in as seen in the last hour of transportation in which two fish died in the treatment group transported for 6hrs.

Antioxidant Profile of Fish Transported in Plastic Bags

The control group's mean SOD values differed significantly (P<0.05) between day 0 and other days with day 0 having the lowest value (22.24± 3.79) while day 6 showed the highest value (177.86± 3.11). The mean values for different transport duration 1hr, 3hrs, and 6hrs significantly increased (P≤0.05) in the subsequent days **(Table 2).**

Table 2: Superoxide dismutase (SOD) parameters of transported fish

Para.	Days	Control	1hr	3hrs	6hrs
SOD	0	22.24^{A} ±3.79	41.59^{A}±0.03	54.57^{A}±6.26	37.14^{A}±1.78
	3	139.07^{B}±28.02	128.05^{B} ±3.78	130.63^{B} ±3.32	129.18^{B} ±21.78
	6	177.86^{B} ±3.11	204.69^{C} ±5.98	173.45^{C} ±3.83	173.50^{C} ±3.55

In each column, values with distinct superscripts are significantly different (P <0.05).

When the control group's mean CAT values were compared to the mean CAT values for each transport duration, a significant difference was observed in the control group (P<0.05) between the day 3 recovery period and the other days. CAT levels in fish transported for 1 hour showed significant improvement (P<0.05) across the days, fish transported for 3 hours differed significantly (P<0.05), while levels in fish transported for 6 hours showed no significant difference (P>0.05) across the different days **(Table 3).**

Table 3: Catalase (CAT) parameter of transported fish

Para.	Days	Control	1hr	3hrs	6hrs
CAT	0	$0.44^{A} \pm 0.01$	$0.45^{A} \pm 0.01$	$0.30^{A} \pm 0.03$	0.85 ± 0.07
	3	$1.83^{B} \pm 0.17$	$1.31^{B} \pm 0.14$	$2.13^{C} \pm 0.03$	1.66 ± 0.44
	6	$0.75^{A} \pm 0.01$	$1.91^{C} \pm 0.20$	$1.39^{B} \pm 0.16$	1.53 ± 0.14

In each column, values with distinct superscripts are significantly different (P <0.05).

The values of GSH for the control group and fish transported for 1hr were significantly lower (P<0.05) on day 0 for control group and 1hr showing the lowest value of (0.32± 0.06) and (0.32± 0.01) respectively. GSH levels in fish transported for 3hrs, and 6hrs significantly increased (P<0.05) across the recovery days with the lowest values recorded on day 0 and higher values on day 3 and 6 **(Table 4).**

Table 4: Reduced glutathione (GSH) parameters of transported fish

Para.	Days	Control	1hr	3hrs	6hrs
GSH	0	$0.32^{A} \pm 0.06$	$0.32^{A} \pm 0.01$	$0.33^{A} \pm 0.01$	$0.04^{A} \pm 0.01$
	3	$0.67^{B} \pm 0.01$	$0.90^{B} \pm 0.01$	$1.01^{C} \pm 0.02$	$0.94^{C} \pm 0.01$
	6	$0.64^{B} \pm 0.01$	$0.88^{B} \pm 0.04$	$0.65^{B} \pm 0.01$	$0.78^{B} \pm 0.01$

In each column, values with distinct superscripts are significantly different (P <0.05).

mRNA Expression of SOD and CAT for Transported Fish in Plastic Bags

The mRNA expression of SOD for fish transported for 1hr,3hrs and 6hrs showed highest values on day 0 and lowest values on day 3 while the control group showed a significant rise (P< 0.05) across the days **(Table 5).**

Table 5: Superoxide dismutase (SOD) mRNA expression over transport duration

Para	Days	Control	1hr	3hrs	6hrs
SOD	0	$1.01^{A}\pm0.05$	$43.44^{C}\pm 0.11$	$11.16^{C}\pm0.22$	$14.44^{C}\pm0.22$
	3	$5.59^{B}\pm0.03$	$1.50^{A}\pm 0.05$	$2.14^{A}\pm0.11$	$1.42^{A}\pm0.17$
	6	$29.55^{C}\pm0.11$	$12.44^{C}\pm0.22$	$6.47^{B}\pm0.15$	$4.25^{B}\pm0.03$

In each column, values with distinct superscripts are significantly different (P <0.05).

Comparing the mean values of CAT across the days for each transport duration and the control group, fish transported for 3hrs showed significant difference (P<0.05) between day 3 and other days while in comparison with the control group, CAT mRNA expression in the transported fish (for 1hr and 6hrs respectively)were significantly different (P< 0.05) across the days **(Table 6).**

Table 6: Catalase (CAT) mRNA expression over transport duration

Para	Days	Control	1hr	3hrs	6hrs
CAT	0	1.00^{A}±0.05	9.17^{C}±0.22	2.26^{A}±0.40	1.23^{B}±0.05
	3	1.55^{B}±0.11	0.84^{A}±0.40	4.12^{C}± 0.03	0.72^{A}±0.05
	6	2.83^{C}± 0.11	3.67^{B}± 0.22	1.48^{A}±0.05	2.06^{C}± 0.11

In each column, values with distinct superscripts are significantly different (P <0.05).

DISCUSSION

Following transportation, it was discovered that some of the fish subjected to transportation stress died over time. Mortality of fish was recorded in recovery tanks of fish transported for 6hrs. No mortality was recorded for the first few hours of transportation in transported fish and the control group (not transported fish). This suggests that survival of *C. gariepinus* juveniles decreased with the duration of transportation in plastic bags. Long periods of transportation may have resulted in mortality, and comparable results have been observed for silver catfish transportation (Golombieski *et al.,* 2003), Channel catfish (Gomes *et al.,* 2000) and *Oreochromis niloticus* (Orina *et al.,* 2014). The survival of *C. gariepinus* juveniles recorded for the first few hours of transportation may be related to their defensive adaptations and *C. gariepinus* hardiness (Ololade and Ogini, 2010). The survival of fish reported in this study is consistent with the findings of Gomes *et al.* (2003), in which they reported no mortality after transportation of Pirarucu with and without oxygen supply and similar to that observed by Onyia and Ladu, (2018) who reported that the transportation of *Oreochromis niloticus* in plastic bags and opened Jerican have no negative effect on the fish survival but plastic Jerican performed better than polythene bag.

In both the transported *C. gariepinus* juveniles and the control group, there was a considerable rise in SOD levels as the number of days increased. Furthermore, the SOD activities of certain transported fish did not differ significantly from those of the control group. The increase in SOD over days implies a spike in superoxide radicals, which helps to keep the radicals in check and protect the integrity of the cellular membrane. Previous study revealed that SOD activity could be enhanced to help cells cope with an increase in reactive oxygen species (ROS) (Zhao *et al.,* 2013). The enhanced SOD activity seen in this study agrees with findings of Rakhmawati *et al.* (2018) in which they reported increased SOD activity in Red Tilapia given feed containing chromium, and Ogueji *et al*. (2020) where they reported an increased SOD activity after exposure of *C. gariepinus* to acute concentration of ivermectin. In contrast, Ogueji *et al*. (2017b) found a decrease in SOD activity in diazepam-treated African catfish, while Ajima *et al*. (2017) found a decrease in SOD activity in Nile tilapia after a sublethal exposure to verapamil. Similarly, Wu *et al*. (2018) reported that Methylmercury inhibited SOD activity in juvenile large yellow croaker.

In the current investigation, a significant rise in catalase activity was seen in both the transported *C. gariepinus* juveniles and the control group as the recovery days increased. The increase in catalase could be an adaptation to protect fish from the toxicity of damaging free radicals caused by transportation. This finding is consistent with reports by Ogueji *et al*. (2020) who observed enhanced catalase activity in Ivermectin-treated *C.gariepinus* juveniles. Wu *et al*. (2018) reported a consistent rise in catalase activity in the juvenile Seabass. In

contrast, *C.gariepinus* juveniles treated to acute ibuprofen and sublethal diazepam showed a reduction in catalase activity (Ogueji *et al.,* 2017a, 2017b).

In both the transported *C.gariepinus* juveniles and the control group, there was a marked increase in GSH levels as the number of recovery days increased. The increased GSH indicates that the experimental fish has recovered from transportation stress, and it could be due to increased amino acid substrate intake as well as biosynthetic enzyme activity in order to protect the fish from oxidative damage. Increased GSH activity in *Carassius auratus* exposed to benzophenone-4 reported by Zhang *et al*. (2018) corroborates findings obtained in this study. GSH levels increased similarly in Croaker juveniles (Wu *et al.,* 2018) and Matrinxa juveniles (Monteiro *et al.,* 2010).

When compared to the control group, SOD mRNA expression was up-regulated on day 0 but down-regulated on day 3 and day 6 for all transportation duration. The rise in hepatic SOD activity found in this work implies that African catfish juveniles can protect themselves from transportation-induced oxidative stress by activating the SOD antioxidant-related gene as a unique adaptational mechanism. This could be due to SOD's potential to scavenge high amounts of ROS and mitigate the oxidative damage caused by transportation. This corroborates the findings of Koner *et al.* (2021), who found that SOD activity increased in the liver of Magur catfish exposed to Zinc-oxide nanoparticle-induced oxidative stress. Under copper ions exposure, SOD was found to increase in the liver of hybrid yellow catfish (Qiang *et al.,* 2019). In comparison to the control group, mRNA expression of CAT activity increased significantly on day 0, reduced on day 3, and increased marginally on day 6. CAT activity rose on days 0 and 3 but reduced on day 6 in fish transported for 3 hours compared to the control group. CAT activity increased on day 0, reduced on day 3, and was not substantially different from the control group on day 6. CAT activity increased on day 0, decreased on day 3, and was not significantly different from the control group on day 6. The activity of CAT in the liver of *C.gariepinus* juveniles increased quickly after transportation (Day 0) but began to decline with longer recovery days, according to this study. This is consistent with the findings of Qiang *et al*. (2019), who found that CAT activity in the liver of hybrid yellow catfish rose during the early stages of copper ion exposure but decreased as exposure time increased. Qiang *et al*. (2017), Xie *et al*. (2018), Jia *et al*. (2011), all found similar results. The ability of *C.gariepinus* juveniles to eliminate excess ROS from the body, leading to reduced oxidative damage caused by transportation stress, may be due to the increase in CAT activity observed in this study. The CAT's breakdown of H_2O_2 may be responsible for this. However, as the stress caused by transportation built up in the body, increased ROS in the liver inhibited the formation of CAT and SOD resulting in reduced enzyme activity.

CONCLUSION

The antioxidant profile and hepatic mRNA expression of transported fish changed as a result of fish transport, according to this study. Some of the transported fish, on the other hand, were able to defend themselves against the oxidative stress caused by transportation. As a result, this research provides insights into the health state of African catfish exposed to a simulated transport system, which would go a long way toward decreasing fish loss and, as a result, increase fish farmers' income and profit.

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