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DETECTION OF RESPIRATORY BURST IN CATLA CATLA (HAMILTON, 1822) FINGERLING UNDERGOING TRANSPORTATION STRESS BY USING NBT TEST

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Abstract

Catla fingerlings' weighing average weight (31.36±1.26 g) was selected for further transportation experiment. Catla catla fingerlings were packed at two densities, 25 g/l (optimum) and 50 g/l (double of the optimum) of ten replicates each for 6 h ,12 h,18 h and 24 h respectively in polyethylene bags of dimensions (L-77.8 cm x B-40 cm), which were filled with 5 liters water, i.e., 1/3rd water and 2/3rd oxygen. The samples were regularly collected at CIFE Aquaculture Wet Laboratory at every 6 h (i.e., 6, 12, 18 and 24 h) intervals for taking blood for estimation of stress parameter i.e. NBT test of catla fingerlings. The vehicle was continuously running for 24 hrs in and around Mumbai from 8.00 A.M to 8.00 A.M. covering a distance of about 640 Km. It was observed that NBT test value got drastically changed is a good indicator of stress during transportation of Catla catla fingerlings packed at high density and increasing transportation duration. Hence, 25 g/l was the optimum packing density of Catla catla fingerlings for 24hrs duration. It was also concluded that increase in packing density resulted in increase of stress. There was a decreasing trend of NBT test value in both packing densities as the duration of time increased. There was a statistical significant interaction between the packing density (i.e., 25g/l and 50g/l) and transportation period (i.e., 0h, 6h, 12h, 18h and 24h) on NBT value . In case of double the optimum density results, it was found that up to 12hrs, this density was optimum and after that the stress parameter NBT value .

Key words: *Catla catla, fingerling, transportation, stress, NBT test*

Introduction

In aquaculture system transportation of live fish often occurred. In course of transportation procedure, fish are exposed to many stressors including high density induced crowding, variation of dissolved oxygen, water temperature, and ammonia nitrogen. Due to these stressors which act on fish and evokes primary responses, such as increases in corticosteroid and catecholamine hormones, secondary responses such as metabolic changes, immune function changes, and tertiary responses such as modified behavioural patterns (Adam,2002.)

Early defence mechanism in multicellular organism based on their innate immunity system which provide them the ability to recognize the pathogen and take action against them

rapidly.(Papermaster etal.,1964 and Alvarez-Pellitero,2008) The most important early defence mechanisms is the respiratory burst which plays important role in eradication of pathogen.and also shown in regeneration of tissues.. In immune system respiratory burst is an important feature The increase in cellular oxygen uptake that marks the initiation of the respiratory burst is followed by the production of reactive oxygen species (ROS) such as superoxide anion and hydrogen peroxide which play a role in the clearance of pathogens and tissue regeneration processes. Therefore, the respiratory burst and associated ROS constitute important indicators of fish health status. Therefore, the respiratory burst is a significant mechanism that can be used to monitor health status in fish. Phagocytes have also been related to the recognition of damage-associated molecular patterns (DAMPs), those being self-signals of tissue damage and cell death(Baianchi ,2007 and Magnadottir,2006) The recognition of all these molecules occurs using special receptors called pattern-recognition receptors (PRRs), and trigger a series of events including the respiratory burst (Baianchi ,2007;Akira and Takeda,2004;Plouffe etal.,2005;Sepulcre etal.,2007)

The initiation of the respiratory burst is marked by an increase in oxygen cellular uptake, followed by the one electron reduction of molecular oxygen (O2) to superoxide anions (O2 -). This reaction is catalysed by the membrane-associated enzyme nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, using NADPH as the electron donor (Ballavite,1988; Babior,1999;Chung and Secombes,1988; Madamanchi and Rung,2007; Pick and Mizel,1988;) . Baehner and Nathan (1968) who introduced the use of nitroblue tetrazolium (NBT) in the detection of respiratory burst. The NBT assay protocol has been optimized over the years but its principle has remained the same (Chung and Secombes, 2007 ; Alfoldy and Lemmel,1979; Boshra etal., 2004; Chettri , 2010 ; Drexhage etal.,1978; El-Boshy etal., 2010 . Rotllan etal., 2004; Schopf, 1984) NBT is a yellow, water soluble substance which is internalized by phagocytes, and then reduced intracellularly to formazan during the respiratory burst. For quantitation, the cell membrane is disrupted, the formazan is dissolved in KOH and the absorbance is read from 509 to 690 nm(Baehner and Nathan,1968; Alfoldy and Lemmel, 1979; Chettri etal., 2010; Drexhage etal., 1978; Schopf etal., 1984) NBT has perhaps become the most popular method for monitoring biological responses to various stimuli through their influence on the respiratory burst (Weber, 1990)

In transportation procedure is broadly categorize into pre transportation (such as collection, grading, netting, air exposure, and packing) and other is actual transportation processes (such as water movement, vibrations, and water condition change)which are stressful to fishes(Paterson etal.,2003 ; Dhanasiri etal.,2013; Pakhira etal.,2015). During transportation, fishes repond with secretion of cortisol hormone which is aprimary stress response (Barton and Iwama,1991). According to Barton and Iwama,1991elevated cortisol level leads to increase in glucose content and there is altered electrolyte homeostasis. Hypoxia, hyperoxia, increased ammonia, and high stocking density leads to oxidative stress in fishes. This type of situation is very much prevalent during transportation of fishes. .(Lushchak,2011;Sahin etal.,2014; Sun etal.,2014).

In Indian subcontinent catla catla fish is one of the preferred fish among the Indian major carps due to its high commercial value, compatible with other carps,high growth rate ,preferred by the consumers,complimentary feeding habit(Laxmappa, 2014). Ctla seed is transported to defecit fish seed area of culture farm ,or from hatchery to the culture farm is most prevalent practice either in open system or in closed system of transportation. During transportation various stressors acts on catla fish seed leads sequential stress responses i.e. primary, secondary and tertiary. Most important tertiary stress response is lowered immune competence of fish seed during the fish transportation which leads to mass mortality or delayed mortality after few days of transportation. This leads to high economic loss of farmer or entrepreneurs. Scanty literature is available on oxidative stress in fishes of Catla fish fingerlings during transportation. This study is an attempt to throw light on how to quantify the oxidative stress response by using **Nitroblue tetrazolium test (NBT)** assay of serum extracted from fingerlings undergone transportation stress. This study helps people to assist in understanding of physiological responses of Catla fingerlings experiencing transportations stress.

Methodology

The fingerlings of *Catla catla* were packed at Aquaculture Division Wet Lab of Central Institute of Fisheries Education, Versova, Mumbai and placed in motorized vehicle for transporation in and around Mumbai for 24 hrs. The sampling of the catla fingerlings was done in Aquaculture Biology Lab.

Experimental fish and their maintenance

Before proceeding to the experiment, *Catla catla* fingerlings were procured from Khar Land Research Station, Panvel of Dr. B. S. K. K. V., Dapoli, Maharashtra, India and were acclimatized for 30 days in 2000 L fibreglass tank at the wet laboratory of Aquaculture Division, Central Institute of Fisheries Education, Versova, Mumbai with proper aeration and 25 percent water replenishment on daily basis. During this acclimatization process, they were fed with 2% of their body weight twice daily with formulated diet containing groundnut oilcake, fishmeal, soybean flour, rice powder, carboxymethyl cellulose, cod liver oil, sunflower oil, vitamin and mineral premix. Water parameters were fortnightly observed and found in optimum range. Feeding was stopped to fingerlings 24 hrs prior to commencement of the transportation experiment.

Experimental design and sampling

Catla fingerlings' body measurements, *i.e.,* average weight and length were taken prior to packaging. Healthy fingerlings weighing average weight (31.36±1.26 g) were selected for further transportation experiment. The fingerlings were packed at five densities, *i.e.,* 25 g/L (T_1), 50 g/L (T_2), 75 g/L (T_3), 100 g/L (T_4) and 125 g/L (T_5) in polyethylene bags of dimensions (L-77.8 cm x B-40 cm), which were filled with 5 liters water, *i.e.,* 1/3rd water and 2/3rd oxygen. These five groups of packing densities were packed in triplicate.

Transportation Protocol

Fishes were packed at different stocking densities, *i.e.,* 25 g, 50 g, 75 g, 100 g and 125 g per litre in triplicate at Aquaculture Wet Laboratory, Central Institute of Fisheries Education, Versova, Mumbai and transported for a period of 24 hrs in a motorized vehicle. The samples were regularly collected at CIFE Aquaculture Wet Laboratory at every 6 h (*i.e.,* 6, 12, 18 and 24 h) intervals for taking various stress parameters of catla fingerlings. The vehicle was continuously running for 24 hrs in and around Mumbai from 8.00 A.M to 8.00 A.M. covering a distance of about 640 Km .

Three fish from each replicate were drawn from these five groups after 6, 12, 18 and 24 h of transportation. Stress indicator like NBT were estimated from serum of fingerlings. It was observed from these estimations that optimum packing density for 6 h, 12 h, 18 h and 24 h transportation of catla fingerlings was 75 g/l, 75 g/l, 50 g/l and 25 g/l

respectively. From the above trials, it was concluded that for 24 h transportation, 25 g/l was optimum packing density for *Catla catla* fingerlings.

Catla catla fingerlings were packed at two densities, 25 g/l (optimum) and 50 g/l (double of the optimum) of ten replicates each for 6 h ,12 h,18 h and 24 h respectively in two sets. Above said transportation protocol was followed. One set of packing was used for determining stress parameters serum biochemical parameter like protein, albumin a,globulin and A:G ratio, which was determined from serum of *Catla catla* fingerlings.

Collection of blood for NBT assay

Blood was collected by puncturing the *Venacaudal,* using a tuberculin medical syringe, which was previously rinsed with 2.7% EDTA solution. Fingerlings were anesthetized with clove oil (MERCK, GERMANY) @ 50 µl per litre of water before taking their blood. Collected blood was then transferred immediately to test tube coated with thin layer of EDTA powder (as an anticoagulant) and shake well in order to prevent haemolysis of blood.

Estimation of Nitroblue tetrazolium test (NBT)

Nitroblue tetrazolium assay was done by the method of Secombes (1996) as modified by Stasiack and Baumana (1996). 100µl of blood was placed into the wells of 'U' bottom micro titre plates and incubated at 37^0C for 1 hr to facilitate adhesion of cells. Then the supernatant was removed and the loaded wells were washed three times with PBS. Having washed, 100µl of 0.2% NBT was added and plate was incubated for further 1 hr. The cells were then mixed with 100% methanol for 2-3 minutes and again washed thrice with 70% methanol. The plates were then air dried. 120 µl 2N KOH and 140 µl dimethyl sulphoxide were added into each well to dissolve the formazon blue precipitate formed. The OD of the turquoise blue coloured solution was then read in ELISA reader at 620 nm.

Physico-chemical parameters of Water

Water quality parameters, *viz,*.temperature, pH (pH meter having the temperature probe), dissolved oxygen by azide modification method (APHA-AWWA-WEF.,1998), free carbon dioxide titrimetric method (APHA-AWWA-WEF,1998), ammonia by spectrophotometerically at 640nm wavelength by phenate method (APHA-AWWA-WEF,1998), nitrite was estimated spectrophotometerically at 543nm wavelength (APHA-AWWA-WEF,1998) and nitrate was estimated spectrophotometerically at 543nm wavelength (APHA-AWWA-WEF.,1998) were recorded in this experiment.

Statistical Analysis

All data obtained were subjected to Two way ANOVA procedure of IBM SPSS(Ver.16.0) and significant means were separated by Tukey's HSD option of same software and further data obtained were subjected to Independent sample t-Test procedure of IBM SPSS(Ver.16.0) and significant means were separated by Tukey's HSD option of same software.

Results

Nitro blue tetrazolium test was conducted by using blood of *Catla catla* fingerlings subjected to different transportation duration in two packing densities are given in Table 1. There was an decreasing trend of NBT values in both packaging densities (*i.e.*, 25 and 50 g/l) as the duration of time increased. The packing density of 25 g/l had high NBT value as compared to 50 g/l. Two way ANOVA revealed that there was a statistical significant interaction ($p<0.05$) between packing densities (25 and 50 g/l) and transportation time, *i.e.*, T_1 (0h), T_2, (6h),T_3 (12h),T_4 (18h) and T_5 (24h) on NBT values. There was a significant difference between packing density and transportation time.

NBT values varied significantly ($p<0.05$) for different transportation durations T_1 and T_2,T_3 ,T_4,T_5 but there was no significant difference between T_2, T_3, T_4,T_5 undertaken in the experiment when the fishes were packed at a density of 25 g/l. However, NBT value was significantly different ($p<0.05$) for the transportation durations T_1 and T_2,T_3,T_4, T_5 ; but there was no significant difference between T_2,T_3 ,T_4, when they were packed at a density of 50g/l.

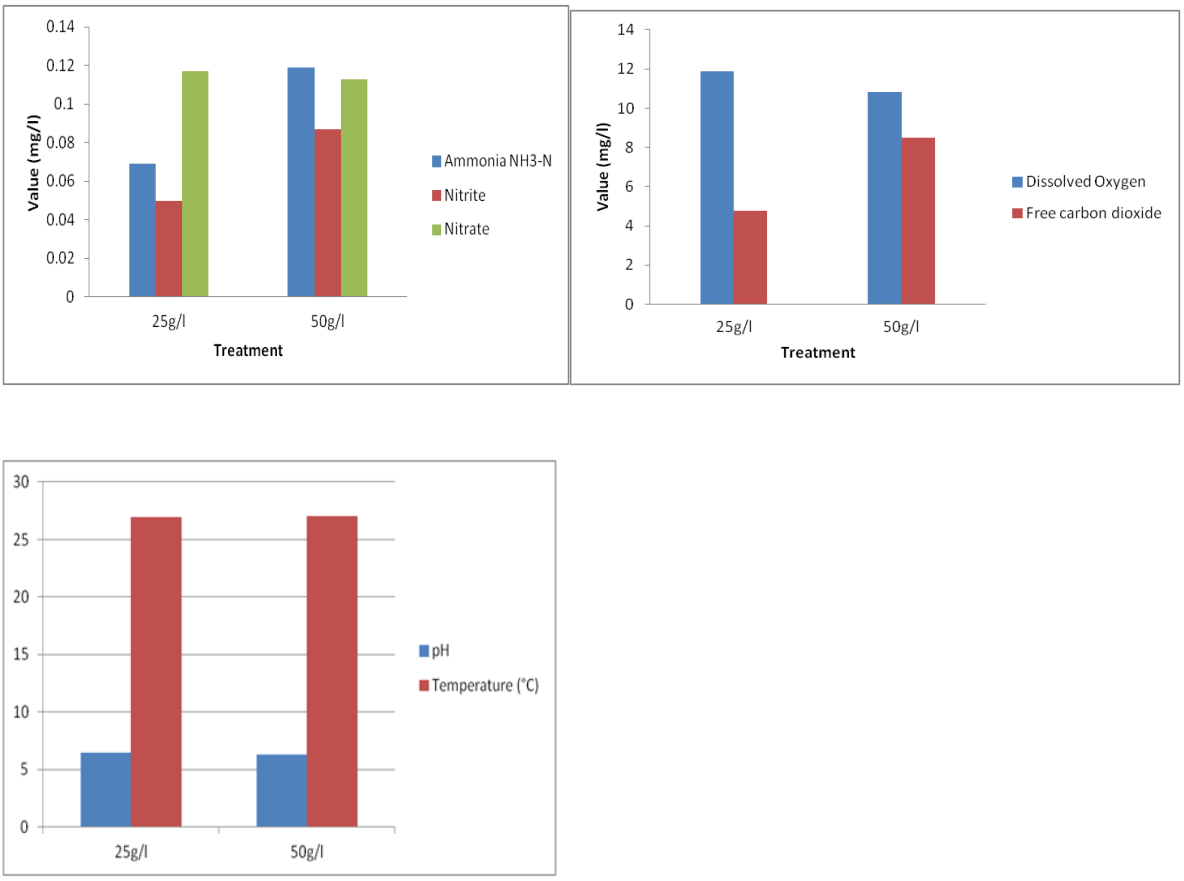
Table 1. Nitro blue tetrazolium test of *Catla catla* fingerlings transported in oxygen inflated plastic bags for varying time period.

Treatment		**NBT**
Packaging density		
25g/l		0.321[a]
50g/l		0.311[b]
SEM		0.001
P-value		S(0.00)
T_1(0h)		0.403[c]
T_2 (6h)		0.303[b]
T_3 (12h)		0.299[b]
T4(18h)		0.281[a]
T_5 (24h)		0.278[a]
SEM		0.002
P-value		S(0.00)
	Packaging Density*Duration	
25g/l	**T_1(0h)**	0.403[c]
	T_2 (6h)	0.306[b]
	T_3 (12h)	0.301[b]
	T4(18h)	0.299[b]
	T_5 (24h)	0.295[b]
50g/l	**T_1(0h)**	0.403[c]
	T_2 (6h)	0.299[b]
	T_3 (12h)	0.297[b]
	T4(18h)	0.294[b]
	T_5 (24h)	0.262[a]
	SEM	0.003
	P-value	S(0.00)

*Treatment means represent the average values of three plastic tubs per treatment. Tukey HSD range test was conducted for treatment means only if there was a significant interaction

(ANOVA, $p < 0.05$). Means value in same column with different superscript differ significantly ($p<0.05$). S-Significant, NS-Nonsignificant.

Fig.1 Water quality parameters after different transportation durations at two packing densities



Discussion

During transportation causes many stressors e.g., hypoxia, hyperoxia, increased ammonia, and high stocking density, can cause oxidative stress (Lushchak,2011; Sahin etal.,2014;Sun etal.,2014). Stress triggers irregular oxidative responses in the aerobic metabolic pathways, which induces the formation reactive oxygen species (ROS). Accumulation of ROS inside cells leads to oxidative stress (OS), resulting in lipid peroxidation, protein carboxylation, and damage of the nucleic acid (Halliwell and Gutteridge,2001). This ROS can be measured by NBT assay using blood of fishes undergone transportation stress. During transportation of catla fingerling there was reduction of NBT value in blood when stocking density increase from 25g/L to 50g/L. and also reduction takes place when transportation duration increases

from 0hrs to 24 hrs. NBT has perhaps become the most popular method for monitoring biological responses to various stimuli through their influence on the respiratory burst (Weber, 1990)

Conclusion

As per above results, it was concluded that the NBT assay of fish blood serum is a stress indicator during transportation. Hence, 25 g/l was the optimum packing density of *Catla catla* fingerlings for 24hrs duration. It was also concluded that increase in packing density and increase in transportation duration leads to decreasing trend of NBT assay which leads increase of stress. There was a statistical significant interaction between the packing density (*i.e.*, 25g/l and 50g/l) and transportation period (*i.e.*, 0h, 6h, 12h, 18h and 24h) on, NBT. There was a decreasing trend of NBT in both packing densities as the duration of time increased. In case of double the optimum density results, it was found that up to 12hrs, this density was optimum and after that the stress parameters got drastically changed.

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