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Note

Effect of packing density on selected tissue biochemical parameters of hatchery produced fingerlings of orange spotted grouper *Epinephelus coioides* (Hamilton, 1822) during transportaion

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ABSTRACT

Effect of different packing densities on water quality parameters, survival and selected tissue biochemical parameters during transportation of hatchery produced fingerlings of orange spotted grouper *Epinephelus coioides* (Hamilton, 1822) was investigated. Fingerlings (weight 3.0±0.2 g and length 6.0±0.2 cm) were packed in sealed double layered oxygen packed polythene bags (water and oxygen ratio 1:3) at different packing densities of 20, 30, 40 and 50 no. l^{-1}. The packed fishes were transported for 6 h. After transportation, water samples and tissue samples from fishes were collected for further analyses. Levels of tissue glucose and selected metabolic enzymes (lactate dehydrogenase, LDH; aspartate amino transferase, AST and alanine amino transferase, ALT) significantly ($p<0.05$) increased with increased packing density. Water quality parameters *viz*., pH, dissolved oxygen, CO_2, alkalinity, total ammonia nitrogen (TAN) and nitrite nitrogen (NO_2-N) were also significantly different at higher packing densities ($p<0.05$). However, levels of all the tissue biochemical parameters tested were in tolerable range and no mortality of fingerlings was recorded at any of the packing densities. Though the tissue enzyme levels were significantly higher and water quality was significantly deteriorated at the highest packing density of 50 no. l^{-1}, it did not lead to mortality of fish. Therefore, it is inferred that this density can be used for short distance transportation of fingerlings of orange spotted grouper.

Keywords: Biochemical parameters, Enzymes, *Epinephelus coioides,* Glucose, Orange spotted grouper, Packing density, Transportation

Groupers, particularly under the genus *Epinephelus* comprises important candidate species for commercial aquaculture in the Asia-Pacific region, due to their consumer preference, robustness under high stocking conditions, good feed conversion rate, high adaptability to different culture systems, rapid growth at eleveated temperatures, salinity tolerance and superior flesh quality. The orange spotted grouper *Epinephelus coioides* (Hamilton, 1822) is a commercially important carnivorous fish with high demand in both local and international markets particularly in south-east Asia and Japan, especially in live fish markets (Kuo, 1995). Earlier, grouper farming was solely dependent on the seeds collected from the wild. The major constraints in the development of orange spotted grouper farming are the inadequate supply of seeds, lack of appropriate handling techniques during collection, transport and storage of collected fish and management of the wild stocks. Difficulties in larval rearing during early stages of groupers have become the major bottleneck hindering the development of mass fingerling production (Kohno *et al*., 1997). Broodstock development, breeding and larval rearing of *E. coioides* has been successfully standardised at Visakhapatnam Regional Centre (VRC) of ICAR-Central Marine Fisheries Research Institute (ICAR-CMFRI).

Fish seed transportation mainly from hatchery to grow-out culture sites is an inevitable procedure. Sealed plastic bags partly filled with water and oxygen is the simplest and widely accepted closed system of transport in which all the requirements for survival are self-contained. In closed systems, slight shaking of the bag supports the mixing of oxygen to water. Transportation without optimum conditions of water volume, oxygen and packing densities may lead to metabolic disturbance and mortalities (Berka, 1986; Ayson *et al*., 1990; Gou *et al*., 1995; Kaiser and Vine, 1998). Lowering the water temperature or application of anesthetics, can reduce the metabolic rate of fish seeds even at higher packing densities (Teo *et al*., 1989; Ayson *et al*., 1990; Kaiser and Vine, 1998). Stressed fish exhibit various physiological responses like fast release of stress hormones *i.e*., catecholamines and cortisol into blood as the primary response (Randall and Perry, 1992) and increased levels of

glucose for providing energy to various tissues under stress (Begg and Pankhurst, 2004). Serum glucose level is the most easily measured inexpensive indicator of secondary phase stress response in fish (Wedemeyer *et al*., 1990). Estimation of water quality, metabolic and biochemical parameters are the ideal indicators of stress.

Published reports on transportation of fish seeds of selected fish species are available (Berka, 1986; Carmichael and Tomasso, 1988; Chow *et al*., 1994; Kaiser and Vine, 1998). However, there is no documented report on transportation of hatchery reared orange spotted grouper (*E. coioides*) fingerlings. In order to avoid stress and water quality related mortality during transportation, packing density needs to be optimised for a specific period of transportation. With this background, an attempt was made to evaluate the levels of selected tissue biochemical parameters and to assess the water quality at various levels of packing density during transportation of fingerlings of *E. coioides*.

Orange spotted grouper fingerlings produced at the mariculture hatchery at VRC of ICAR-CMFRI were used for the study. The fishes were conditioned without feed for 12 h before packing to reduce the faecal out put and metabolic activity.

Fingerlings (average weight 3.0±0.2 g; length 6.0±0.2 cm) were packed in sealed double layered polythene bags (71.25 x 36.5 cm), filled with water and oxygen at ratio 1:3. Fish fingerlings were stocked at four different densities *viz.*, 20, 30, 40 and 50 no. l^{-1} in triplicates. Initial dissolved oxygen level was brought to 25.5 mg l^{-1} with pure O_2 supplied from oxygen cylinder before transportation. These packets were further placed in thermocoal boxes with gel ice packets (precooled at -10^{0}C) to reduce the temperature during transportation. The packed fishes were transported by road from the mariculture hatchery at VRC of ICAR-CMFRI to a coastal pond located at Pentakota Village, Payakaraopeta Mandal of Visakhapatnam District, Andhra Pradesh. The total duration of transportation was 6 h. On arrival at the site, water as well as fish samples (n=6) were collected from each packing density for estimation of water quality and tissue biochemical parameters. For estimating survival rate (%), fishes were counted from each packet on arrival at destination. Fingerlings maintained in the hatchery before packing were considered as control fishes for analyses.

Water quality parameters like temperature and pH were determined using mercury thermometer and pH meter (Eutech Instruments, pH 700) respectively. Dissolved oxygen (Winkler's method), free CO_2 and alkalinity were measured by titrimetric methods (APHA, 2012). Total ammonia nitrogen (TAN) and nitrite nitrogen (NO2-N) were measured by standard spectrophotometric methods (APHA, 2012).

Fishes were anaesthetised with phenoxy ethanol (50 µl l^{-1}), sacrificed and 10% tissue (muscle) homogenate was prepared in distilled water for glucose estimation and in 250 mm sucrose for the estimation of tissue enzyme levels. The homogenate was centrifuged (5,000 *g* at 4^{0}C for 20 min) and the supernatent was stored at -20^0 C for furthur estimation of tissue glucose and selected metabolic enzyme levels.

Glucose: For analysis of glucose (Nelson, 1944; Somogyi, 1945, 1952), the homogenates were deproteinised with zinc sulphate and barium hydroxide, filtered and the supernatant was placed in test tube, alkaline copper sulphate was added and the tubes were placed in boiling water for 20 min. Arsenomolybdate reagent was added to the test tubes after cooling and absorbance was recorded at 540 nm against a blank.

Lactate dehydrogenase (LDH) (L-Lactate NAD+ oxidoreductase; E.C.1.1.1.27): The LDH activity was assayed by the method of Wroblewski and Ladue (1955). The total 3 ml of the reaction mixture comprised 2.7 ml of 0.1 M phosphate buffer (pH 7.5), 0.1 ml NADH solution (2 mg NADH dissolved in 1 ml phosphate buffer), 0.1 ml homogenate and 0.1 ml sodium pyruvate. OD was recorded at 340 nm at 30 s intervals. The LDH activity was expressed as units mg protein^{-1} min^{-1} at 25^0 C where 1 unit was equal to Δ0.01OD min^{-1}.

Aspartate amino transferase (AST) (L-aspartate: 2 oxaloglutarate aminotransferase, E.C.2.6.1.1): The AST activity was assayed as described by Wootton (1964). The substrate comprised 0.2 M D, L- aspartic acid and 2 mM α-ketoglutarate in 0.05 m phosphate buffer (pH 7.4). In the experimental and control tubes, 0.5 ml of substrate was added. The reaction was started by adding 0.1 ml of homogenate. The assay mixture was incubated at 37^{0}C for 60 min. Reaction was terminated by adding 0.5 ml 1 mM 2, 4 dinitrophenyl hydrazine (DNPH). In the control tubes, the enzyme source was added after adding DNPH solution. The tubes were held at room temperature for 20 min with occasional shaking. Then 5 ml of 0.4 ml NaOH solution was added and the contents were thoroughly mixed. After 10 min, the OD was recorded at 540 nm against blank.

Alanine amino transferase (ALT): (L-alanine: 2 oxaloglutarate aminotransferase; E.C.2.6.1.2): The method described by Wootton (1964) was employed for estimation of ALT activity. The substrate comprised 0.2 M D, L-alanine and 2 mM a-ketoglutarate in 0.05 M phosphate buffer (pH 7.4) and the protocol as described for AST was followed.

Quantification of protein was carried out using Lowry's method (Lowry *et al.*, 1951). Tissue homogenate (0.1 ml) was taken and precipitated using 1 ml of 10% TCA. The protein residue was obtained by discarding the supernatant produced after centrifugation at 5000 *g* for 20 min. The residue was dissolved in 0.5 ml 0.1 N NaOH. An amount of 0.1 ml of the dissolved protein residue was used for further analysis. Alkaline copper sulphate (5 ml) was added and left for 10 min. To this, 1N Folin's reagent was added and incubated for 30 min in the dark. OD was recorded at 660 nm against the blank. Bovine serum albumin was used as standard. All the colourimetric assays were carried out using UV-VIS spectrophotometer (BioTek, USA).

The data were statistically analysed using statistical package SPSS version 16. Comparison among all the treatments was done by one way ANOVA. Comparison between two treatments was done by Duncan's multiple range test (DMRT). Differences were considered statistically significant if $p<0.05$.

Water quality and tissue biochemical parameters before and after transportation of 3 g size fingerlings of the orange spotted grouper were evaluated and presented in Table 1. In the hatchery, water quality parameters recorded just before packing were: temperature - 28.5^{0}C, dissolved oxygen - 3.5 mg l^{-1}, pH - 7.5, alkalinity - 113 mg $CaCO_3$ l^{-1}, CO_2-nil, total ammonia nitrogen (TAN) - nil and nitrite nitrogen (NO_2-N) - nil. After transportation, the dissolved oxygen levels recorded were found to be low at higher packing densities and significantly ($p<0.05$) low dissolved oxygen (2.6±0.1 mg l^{-1}) was recorded in packs stocked at the highest density of 50 no. l^{-1}. This is mainly due to the increased oxygen consumption by the increased number of fingerlings packed during transportation. Packing density and duration of transport influenced the water quality and oxygen consumption (Chatterjee *et al*., 2010). Oxygen consumption by fish depends on stress, water temperature, pH and concentration of carbon dioxide and metabolic products like ammonia. Oxygen consumption generally increases with increase in water temperature (Piper *et al*., 1982). Stress during transportation, also might have led to higher oxygen consumption. Fish need large amount of oxygen to counteract their excitement at first hour of packing during their acclimatisation (Berka, 1986).

Water pH varied from 7.8-6.3 and significantly lowest pH ($p<0.05$) was recorded at the highest packing density. After transportation, significantly ($p<0.05$) increased CO_2 concentrations were recorded at higher packing densities of 40 and 50 no. l^{-1}. CO_2 production through respiration of the animal increases with increased packing density, leading to lowering of pH as reported by Wurts and Durborow (1992). Temperature of water increased with increased packing density during transportation and significant ($p<0.05$) increase was recorded in packs with density of 50 no. l^{-1} (34.5±0.5^{0}C) (Table 1). Temperature beyond optimum limits for a particular species adversely affects its health by increasing their metabolic rates which in turn results in increased levels of toxic ammonia and subsequent oxygen demand. Pramod *et al.* (2010) reported that increase in water temperature accelerated the metabolic rate and consequently the oxygen consumption in *Puntius filamentosus*.

There was an increase in alkalinity during transportation (136-150 mg $CaCO_3$ l^{-1}) which was evident from decreased pH at higher packing densities. Ammonia released by fish into water reacts with water molecules to form ammonium (NH4+) and hydroxyl (OH-) ions and further, the hydroxyl ions react with CO_2 to produce HCO_3- which results in increase of alkalinity (Boyd, 1990). Total ammonia nitrogen (TAN) content increased with increased fish density during transportation and significantly higher level ($p<0.05$) was observed at 50 no. l^{-1}. This might be due to the high energy demand of fish during transportation which led to the direct deamination of important tissue energy sources for energy production resulting in increased ammonia level in fish (Philip and Rajasree, 1996; Smutna *et al.,* 2002). Nitrite concentration increased with increased packing density during transportation (0.003-0.025 mg l^{-1}) but it was within the tolerable limit of the grouper fingerlings in the present study. NO_2-N

Table 1. Water quality parameters (mean ± SE) before and after transportation of grouper fingerlings at different packing densities

Parameters	Before transportation	After transportation			
		20 no. l^{-1}	30 no. l^{-1}	40 no. l^{-1}	50 no. l^{-1}
Dissolved oxygen (mg l^{-1})	25.5±0.05^c	7.71±0.05^d	5.75±0.05^c	4.25±0.05^b	2.6±0.1^a
pH	7.85±0.05^d	7.45±0.05^c	6.75±0.05^b	6.55±0.05^b	6.3±0.1^a
CO_2 (mg l^{-1})	0±0^a	2.06±0.04^b	2.87±0.06^c	3.21±0.02^d	3.26±0.01^d
Temperature (^{0}C)	28.5±0. 5^a	31.5±0.5^b	32.5±0.5^b	33.5±0.5^c	34.5±0.5^d
Alkalinity (mg $CaCO_3$ l^{-1})	113.2±1.0^a	136.5±1.5^b	143.5±1.5^c	141±1.0^c	150.5±0.5^d
TAN (mg l^{-1})	0.001±0.0003^a	1.63±0.005^b	1.63±0.005^b	1.82±0.02^c	2.16±0.03^d
NO_2-N (mg l^{-1})	0.001± 0^a	0.003±0.001^a	0.0115±0.001^b	0.0155±0.001^b	0.025±0.001^c

Values sharing different superscripts differ significantly ($p<0.05$)

concentration recorded in this study was much lower and there was no mortality in any of the packing densities.

The muscle glucose levels significantly ($p<0.05$) increased with increase in packing density of the fingerlings during transportation (Fig. 1). The mean glucose level increased from 33.81 to 48.18 mg dl^{-1} in the highest packing density (50 no. l^{-1}). There was no significant difference ($p>0.05$) in the glucose levels of fingerlings before and after transportation in packing densities of 20 and 30 no. l^{-1}. Similar reports are available with other fish species wherein, increased blood/whole body glucose levels recorded due to various stress factors like transportation, stocking, duration of confinement and handling (Specker and Schreck, 1980; Iverson *et al.,* 1998; Perez-Casanova *et al.,* 2008). Elevated glucose levels are a result of the difference in respiration and other activities during stressed conditions (Ghosh, 1987). Significant ($p<0.05$) increase in glucose levels were observed in fingerlings packed at densities of 40 and 50 no. l^{-1}, but the ranges were within tolerance limits and there was 100% survival of fingerlings at all packing densities.

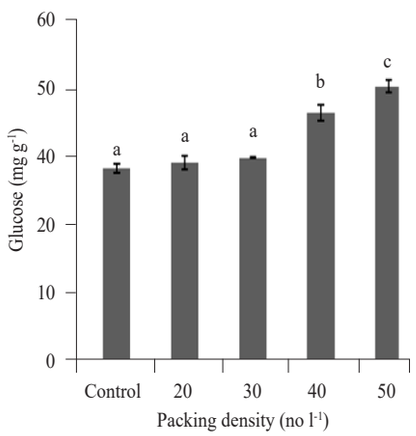

Fig. 1. Muscle glucose content of grouper fingerlings before (Control) and after transportation

Lactate dehydrogenase (LDH) activity of fingerlings varied significantly ($p<0.05$) between before (control) and after transportation in all the packing densities (Fig. 2). The whole body LDH activity increased significantly ($p<0.05$) at packing density of 50 no. l^{-1} than that of 20, 30 and 40 no. l^{-1}. LDH, the terminal enzyme of the glycolytic pathway converts pyruvate to lactate and shows higher activity in aerobic tissues like muscle during low oxygen tension and in the liver during gluconeogenesis (Verma *et al.,* 2007). The increased activity of LDH at the higher packing density could be attributed to the production of lactate, resulting from oxygen limited condition caused due to higher packing density leading to increased gluconeogenesis (Verma *et al.,* 2007).

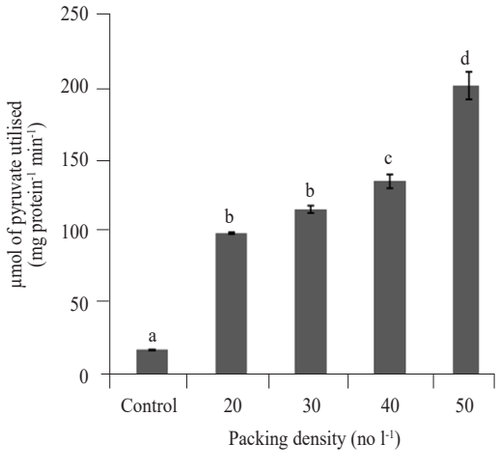

Fig. 2. LDH activity of grouper fingerlings before and after transportation at different packing densities

AST and ALT activity of fingerlings transported at different packing densities increased significantly ($p<0.05$), when compared to control. Highest activities of AST (23.95±0.11 nmoles oxaloacetate mg $protein^{-1}$ min^{-1}) and ALT (44.64±0.55 nmoles pyruvate mg $protein^{-1}$ min^{-1}), were recorded in fingerlings transported at 50 no. l^{-1}, which were significantly ($p<0.05$) different from fingerlings at other packing densities (Fig. 3 and 4). Similar results were observed in common carp (Dobsikova *et al*., 2009) and rohu fingerlings (Chatterjee *et al*., 2009). Fishes utilise protein (amino acids) and lipid for energy fulfillment during stress conditions (Demeal, 1978). Increase in activities of enzymes of protein metabolism (AST and ALT), means mobilisation of aspartate and alanine to oxaloacetate and pyruvate respectively, for the production of glucose to recover the energy during conditions of stress. The end products, oxaloacetate and pyruvate are further utilised as substrates for gluconeogenesis (Chatterjee *et al*., 2006).

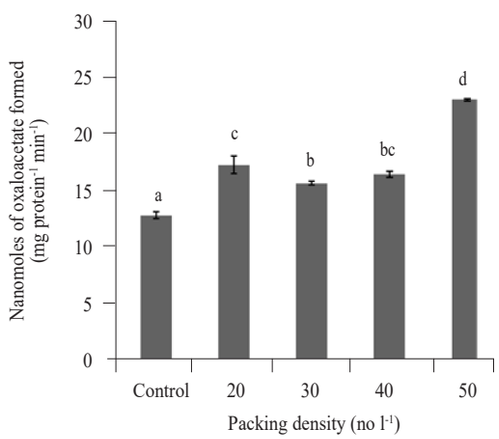

Fig. 3. AST activity of grouper fingerlings before (control) and after transportation at different packing densities

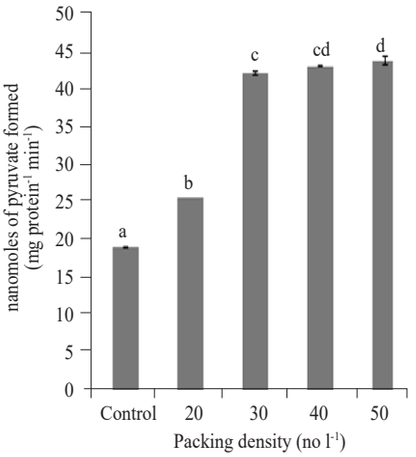

Fig. 4. ALT activity of grouper fingerlings before (control) and after transportation at different packing densities

Results of the present study suggest that transportation of grouper fingerlings at higher packing densities causes stress, which was evident from the water quality as well as tissue biochemical parameters. However, in this study, all the tissue biochemical parameters tested and water quality parameters were found to be in tolerable range, as none of the measured parameters led to mortality of the fingerlings during transportation. Though biochemical parameters were significantly higher and water quality was significantly hampered at the highest packing density of 50 no. l^{-1}, it did not lead to mortality of the transported grouper fingerlings and therefore, it could be suggested that this density can be used for short distance transportation of orange spotted grouper fingerlings.

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