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Effect of stocking density and journey length on the welfare of rohu (*Labeo rohita* Hamilton) fry

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Abstract The effect of higher packing density and increased duration of transport on the survival and key metabolic enzymes of *Labeo rohita* fry was investigated. *L. rohita* fry (length 40 ± 5 mm, weight 0.60 ± 0.13 g) were packed in two different densities 40 and 80 g/l and sampled at 0, 12, 24, and 36 h after packing. Results showed that packing density and length of confinement severely affected the survival of the fry. The whole-body glucose level and the activities of the enzymes, lactate dehydrogenase (LDH), malate dehydrogenase (MDH), glucose-6-phosphatase (G6Pase), fructose-1, 6-bisphosphatase (FBPase), aspartate amino transferase (AST), alanine amino transferase (ALT), and adenosine triphosphatase (ATPase) assayed from the fish whole-body significantly ($P < 0.05$) increased due to increase in the length of the confinement. However, acetylcholine esterase (AchE) activity decreased significantly ($P < 0.05$) with increase in the length of confinement. Similarly, higher packing density also significantly ($P < 0.05$) increased the glucose level and activities of all these enzymes (except AchE). The results revealed that both higher packing density and increased transportation duration mobilize protein resources for glucose production via gluconeogenesis and subsequently activate the glycolysis pathway for energy. The rise in the ATPase activity indicates disruption of the osmoregulatory function and the role of this enzyme in ameliorating it. Overall results suggest that normally practiced packing density of 40 g/l is optimum up to 24-h duration for seed transportation.

Keywords Transportation · Packing density · Metabolic enzymes · Stress

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Introduction

Rohu (*Labeo rohita*) is one of the main carps produced in India both intensively and semi-intensively. Its seeds are transported over long and short distances in India, making seed packing and transportation an integral part of its aquaculture practice. During the entire process of transportation, the seeds are exposed to various stressors like netting, handling, crowding, and confinement and this often results in high mortality either during or after transportation (Singh et al. 2004). Since packing density and duration of transport are two parameters that can be varied, successful transportation aims at ensuring maximum survival at an optimum packing density for a specified duration (Carmicheal 1984). Literature on the transportation of Indian and exotic major carps has specified various packing densities for different duration (Alikunhi 1957; Ramachandran 1969); however, on-site farmers and hatchery managers often decide the packing density based upon the size, duration, and mode of transport.

The stress axis in fish acts through the hypothalamus pituitary chromaffin axis and the hypothalamus pituitary interrenal axis which, respectively, stimulate the production of catecholamines and cortisol. Catecholamine activates glycogenolysis and cortisol gluconeogenesis resulting in increased production of glucose which is needed to combat stress (Pickering 1993; Schreck 1996).

Although the effects of packing density and transportation stress on the physiological responses, bacterial density, and growth of *L. rohita* fingerlings have been investigated (Hasan and Bart 2007), the effects of transportation stress on metabolic enzyme activities are lacking (especially on *L. rohita*). Therefore, in the present study, the effects of transportation stress on the biochemical variable mainly glucose and on the activities of selected enzymes of glycolysis, gluconeogenesis, protein metabolism, membrane transport, and neurotransmission were elucidated.

Materials and methods

Experimental animals

Prior to initiation of the experiment, *L. rohita* fry procured from Khopoli fish seed farm, Maharastra, India, were maintained for 30 days in two 1,000-l fiberglass tanks at the wet laboratory of the Central Institute of Fisheries Education, Mumbai, India, with proper aeration. During this period, they were fed at 2% body weight, twice daily with a formulated diet containing groundnut oil cake, fish meal, rice bran, binder, and vitamin–mineral mixture. Manual water exchange (10%) was done every day. Water quality parameters like temperature, dissolved oxygen, and pH were checked daily and were found within the optimum range. Feeding was stopped 24 h before the start of the experiment.

Experimental design and sampling

Fish were netted using a hand-net from the tanks, and the average weight (0.50 ± 0.13 g) and length (40.00 ± 5.00 mm) were determined by taking the weight and length of 10 fish drawn randomly. The suggested packing density for 40-mm *L. rohita* fry for 12-h duration is 100–120 fry/l, for 24-h is 60–80 fry/l, and a transportation of more than 24-h duration is not practicable for this fish size (personal communication with hatchery managers). In the present study, two packing densities were chosen: one an optimum density (80 fry/l) for

24 h, and the other double the optimum density (160 fry/l). Since the average weight of the fry in the present experiment was 0.5 g, the above-mentioned packing densities in terms of biomass were referred as 40 and 80 g/l, respectively. Both the groups were packed in the polyphone bags (45 cm × 30 cm) containing 1-l water with three replicates each. The air in the bags was then squeezed out and replaced with medical grade oxygen before the bags were immediately tied using a rubber string. Samples (12 fish at every sampling, four fish from each replicate) were drawn from both the groups at 0, 12, 24, and 36 h of packing. Fish were anesthetized using clove oil (50 µl/l), sacrificed, and then a tissue homogenate (10%) was prepared in distilled water for glucose estimation and in 0.3M sucrose for enzyme assay as previously described (Chatterjee et al. 2006; Norouzitallab et al. 2009). The homogenate was centrifuged at 5,000*g* at 4°C for 5 min, and supernatant collected and stored at −20°C for further analysis.

Glucose estimation

For whole-body glucose analysis, the homogenates were deproteinized (v/v) with zinc sulfate and barium hydroxide, centrifuged at 5,000*g* for 5 min, and the supernatant was used for the estimation of glucose according to the method described by Nelson and Somogyi (1945). The supernatant was placed in a test tube, alkaline copper sulfate was added, and the tube was placed in a boiling water bath for 20 min. The test tube was then cooled to room temperature, arsenomolybdate reagent was added, and absorbance was recorded at 540 nm against a blank.

Enzyme assay

Lactate dehydrogenase (LDH; EC 1.1.1.27) was assayed using 0.2 mM NADH solution in 0.1 M phosphate buffer (pH 7.5) and tissue homogenate. The reaction was initiated by adding 0.2 mM sodium pyruvate as the substrate, and OD was recorded at 340 nm for 3 min at an interval of 30 s (Wroblewski and Ladue 1955). A similar reaction mixture was used for the estimation of malate dehydrogenase (MDH; EC 1.1.1.37) except for the substrate, 0.1 mM oxaloacetate (Ochoa 1955). Activity of fructose-1, 6-bisphosphatase (FBPase; EC 3.1.3.11) in the tissue was assayed by the method outlined by Freeland and Harper (1959). The assay solution comprised of 50 mM borate buffer (pH 9.5), 50 mM fructose-1,6-diphosphate (pH 7–7.3) as substrate, 0.5 M $MgSO_4$, and tissue homogenate. The mixture was incubated at 37°C for 30 min, and the reaction was terminated by addition of 10% trichloroacetic acid (TCA) solution. Phosphate liberated was estimated at 600-nm absorbance (Fiske and Subbarow 1925). The glucose-6-phosphatase (G6Pase; EC 3.1.3.9) was assayed by the method described by Marjorie (1964). The assay mixture consisted of malate buffer (pH 6.5), 0.1 M glucose-6-phosphate solution (substrate), and the tissue homogenate. The mixture was incubated at 37°C for 15 min, and the reaction was terminated by addition of 10% TCA solution. Phosphate liberated was estimated as described above. Total adenosine triphosphatase (ATPase; EC 3.6.1.3) was assayed in a reaction mixture of 0.1 M Tris–HCl buffer (pH 7.8), 100 mM NaCl, 20 mM KCl, 3 mM MgCl2, and 5 mM ATP. The mixture was incubated for 15 min, and the reaction was terminated by means of 10% TCA (Post and Sen 1967). Phosphate liberated was estimated at 660 nm (Fiske and Subbarow 1925). Alanine amino transferase (ALT; EC 2.6.1.2) was assayed using 0.2 M DL-alanine and 2 mM L-ketoglutarate in 0.05 M phosphate buffer (pH 7.4) as the substrate and aspartate amino transferase (AST; EC 2.6.1.1) using 0.2 M DL-aspartic acid and 20 mM L-ketoglutarate in 0.05 M phosphate buffer (pH 7.4) as the

substrate, and both were estimated at optical density (OD) of 540 nm (Wootton 1964). Acetylcholine esterase (AchE; EC 3.1.1.7) was assayed by the method described by Hestrin (1949), using a mixture of 0.07 M phosphate buffer (pH 7.2), 4 mM acetylcholine (pH 4.0), and a substrate–buffer mixture (1/10 dilution). The mixture was incubated at 37°C for 30 min. Alkaline hydroxylamine solution was used to terminate the reaction, and HCl (2:1) was added. The color developed by the addition of 10% $FeCl_3$ was measured at 540 nm. All enzyme activities are measured as unit activity/mg protein Total protein content was analyzed from the supernatant (Lowry et al. 1951) for calculating enzyme activities. All the colorimetric assays were carried out using UV–VIS spectrophotometer (E-Merck, Germany).

Statistical analysis

Time-dependent relation on the tested parameters (Glucose, LDH, MDH, FBPase, G6Pase, AST, ALT, ATPase, and AchE) in each group was investigated by one-way ANOVA using SPSS version 11.0. *Post hoc* comparison of the means was carried out using Duncan's multiple range test. Student's *t*-test was applied to test the difference between the packing densities. Differences were considered statistically significant when $P < 0.05$.

Results

There was an apparent effect of packing density (40 or 80 g/l) on the survival of *L. rohita* fry (Fig. 1). The survival of the fry packed at 40 g/l density for 12-, 24-, and 36-h duration was significantly higher ($P < 0.05$) than that in the 80 g/l packing density. Lowest survival was noted in the group packed at 80 g/l density for 36 h. Packing density also had a significant ($P < 0.05$) effect on the whole-body glucose level (Fig. 2). The glucose levels of the fry in both the packing densities increased significantly ($P < 0.05$) with increase in the length of confinement (0, 12, 24, and 36 h). The glucose levels in the 80 g/l packing

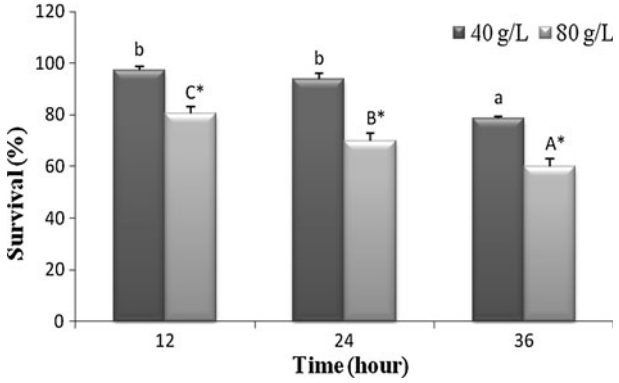

Fig. 1 Survival (%) of *Labeo rohita* fry packed at different densities for increased length of duration. Data are expressed as mean ± SE ($n = 3$). Bar with different alphabet letters (a and b for 40 g/l packing density, A, B, and C for 80 g/l packing density) differs significantly (Duncan's multiple range test, $P < 0.05$). * Indicates significant difference between two packing densities at a particular sampling time ($P < 0.05$, Student's *t*-test)

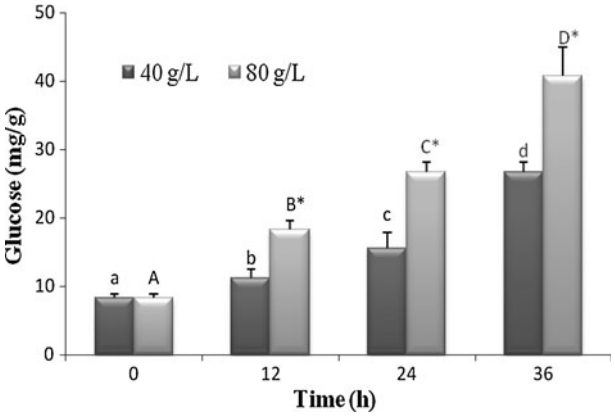

Fig. 2 Whole-body glucose content of *Labeo rohita* fry packed at different densities for increased length of duration. Data are expressed as mean ± SE ($n = 6$). Bar with different alphabet letters (a, b, c and d for 40 g/l packing density, A, B, C, and D for 80 g/l packing density) differs significantly (Duncan's multiple range test, $P < 0.05$). * Indicates significant difference between two packing densities at a particular sampling time ($P < 0.05$, Student's *t*-test)

density at 12, 24, and 36 h were significantly ($P < 0.05$) higher than those in the 40 g/l packing density at the respective time.

Data on the effect of packing density and increased length of confinement on the enzymatic activities of various metabolic pathways are summarized in Table 1. The LDH activity in both the experimental groups significantly increased ($P < 0.05$) from 0 to 12 h and from 24 to 36 h. The enzyme activity in the 80 g/l packing density group was significantly higher ($P < 0.05$) than its 40 g/l counterpart both at 24 and 36 h. The MDH activity in relation to time showed similar trend as LDH. However, no significant ($P > 0.05$) difference was observed between the two packing densities in any of the sampling time.

The activity of the gluconeogenic enzymes, FBPase and G6Pase, increased significantly ($P < 0.05$) with increase in length of confinement period. The FBPase activity in the 80 g/l packing density group was significantly higher ($P < 0.05$) than the 40 g/l packing density group at 12, 24, and 36 h. In case of G6Pase, the difference in activity between the two packing densities was significant (i.e., higher in 80 g/l group) at 12 and 36 h after packing ($P < 0.05$).

The activity of the transaminase enzymes, AST and ALT, showed an increasing trend with increase in the time of confinement as well as due to higher packing density. The increase was significant ($P < 0.05$) between all the sampling times in both the experimental groups for both the enzymes. Significantly higher activity ($P < 0.05$) in the 80 g/l group was observed at 12 h for AST and at 12, 24, and 36 h for ALT.

The enzyme of membrane transport, ATPase, also increased significantly ($P < 0.05$) with increase in the length of confinement period in both the packing densities. The activity of this enzyme in the 80 g/l group was significantly ($P < 0.05$) higher than the 40 g/l group at 24- and 36-h post packing.

The activity of neurotransmission enzyme, AchE, decreased significantly ($P < 0.05$) with the increase in the time of confinement. The activity was lower in the higher packing density (80 g/l) group than that in the 40 g/l group at 12 and 24 h.

Table 1 Effect of packing densities and increased length of confinement on the enzymes activity[1] of anaerobic pathways (lactate dehydrogenase, LDH), glycolytic pathway (malate dehydrogenase, MDH), gluconeogenic pathway (fructose-1,6-bisphosphatase, FBPase, and glucose-6-phosphatase, G6Pase), protein metabolism (aspartate amino transferase, AST, and alanine amino transferase, ALT), membrane transport (adenosine triphosphatase, ATPase), and neurotransmission (acetylcholine esterase, AchE) in the whole-body of *Labeo rohita* fry

Enzymes	Packing density (g/l)	Time (h)			
		0	12	24	36
LDH	40	109.14 ± 6.60^{a}	243.74 ± 12.15^{b}	253.88 ± 11.09^{b}	307.15 ± 19.50^{c}
	80	109.14 ± 6.60^{a}	267.84 ± 33.70^{b}	300.2 ± 15.70^{b}*	415.83 ± 21.80^{c}*
MDH	40	165.16 ± 12.35^{a}	310.34 ± 30.53^{b}	355.89 ± 16.57^{b}	469.67 ± 27.70^{c}
	80	165.16 ± 12.35^{a}	365.88 ± 15.43^{b}	366.89 ± 26.26^{b}	540.56 ± 61.02^{c}
FBPase	40	0.12 ± 0.02^{a}	0.25 ± 0.03^{b}	0.36 ± 0.03^{c}	1.04 ± 0.06^{d}
	80	0.12 ± 0.02^{a}	0.75 ± 0.04^{b}*	0.77 ± 0.05^{b}*	1.83 ± 0.09^{c}*
G6Pase	40	0.11 ± 0.01^{a}	0.18 ± 0.02^{b}	0.38 ± 0.03^{c}	0.60 ± 0.07^{d}
	80	0.11 ± 0.01^{a}	0.29 ± 0.09^{b}*	0.46 ± 0.12^{c}	0.75 ± 0.08^{d}*
AST	40	23.05 ± 2.43^{a}	32.36 ± 3.25^{b}	46.00 ± 2.87^{c}	61.01 ± 3.90^{d}
	80	23.05 ± 2.43^{a}	45.41 ± 3.74^{b}*	52.88 ± 4.48^{c}	68.63 ± 5.97^{d}
ALT	40	0.44 ± 0.096^{a}	1.45 ± 0.52^{b}	4.62 ± 0.54^{c}	7.36 ± 0.98^{d}
	80	0.44 ± 0.09^{a}	18.48 ± 3.26^{b}*	20.2 ± 1.66^{c}*	27.23 ± 2.89^{d}*
ATPase	40	0.95 ± 0.06^{a}	1.35 ± 0.07^{b}	1.72 ± 0.09^{c}	2.12 ± 0.12^{d}
	80	0.95 ± 0.06^{a}	1.39 ± 0.21^{b}	2.88 ± 0.34^{c}*	3.44 ± 0.39^{d}*
AchE	40	6.00 ± 0.48^{a}	2.07 ± 0.46^{b}	1.39 ± 0.20^{a}	1.21 ± 0.14^{a}
	80	6.00 ± 0.48^{c}	1.52 ± 0.10^{b}*	1.22 ± 0.09^{ab}*	0.87 ± 0.18^{a}

Data are expressed as mean ± SE ($n = 6$). Different superscript lower case letters (a, b, c) in the same row indicate significant difference among different time interval in each packing density (Duncan's multiple range test, $P < 0.05$). * Indicates significant difference between two packing densities at a particular sampling time ($P < 0.05$, Student's *t*-test). [1] Enzyme activities are expressed as follows: LDH as μmol of pyruvate utilized/mg protein/min, MDH as μmol of oxaloacetate utilized/mg protein/min, FBPase and G6Pase as mg phosphorus released/min/mg protein, AST as nmol of oxaloacetate released/mg protein/min at 37°C, ALT as nmol of sodium pyruvate released/mg protein/min at 37°C, ATPase as μg of phosphorus/mg protein/min at 37°C, and AchE as μmol of acetyl choline hydrolyzed/mg protein/min at 37°C

Discussion

Transportation of fish seeds from hatchery to grow-out ponds is stressful, yet it is an inevitable procedure in aquacultural practices. Optimizing the packing density for a specified period of transportation is needed to avoid stress-related mortality both during and/or after transportation. Metabolic markers like cortisol (Hasan and Bart 2007) and enzymes (Chatterjee et al. 2006) have been proved as ideal indicators of stress and therefore can provide the base for optimizing the packing density of IMC seed.

In the present study, survival data of the *L. rohita* fry during the three sampling intervals (12, 24, and 36 h) indicate that the fish packed at higher density for 36 h duration were severely stressed. Increased mortality in relation to time in both the packing densities and comparatively higher mortality in the higher packing density indicate stress both due to increased length of confinement and higher packing density and is in close agreement with the results of Gomes et al. (2006), who observed high mortality of tambaqui, *Colossoma macropomum*, juvenile due to transportation stress.

Stress has been known to affect many aspects of the fish physiology (Montero et al. 1999). Like other vertebrates, stressed fish exhibit a series of physiological responses. The primary responses are mediated by the fast release of stress hormones, catecholamine and cortisol, into the fish circulatory system. Catecholamine increases glycogen mobilization (Vijayan et al. 1993), and cortisol affects the gluconeogenesis in fish. The increase in the concentration of glucose with stress assists the animal by providing an energy substrate to various tissues, in order to cope with the increased energy demand. Glucose levels were found to increase both in response to higher packing density and increased length of confinement during this study, indicating an increased energy demand caused by stress. Similar increases in the blood/whole-body glucose level in response to various stressors such as, transportation, confinement, and handling have also been reported in other fish species (Specker and Schreck 1980; Iverson et al. 1998; Pérez-Casanova et al. 2008).

Several studies have shown alteration in the activities of enzymes related to cellular energy metabolism due to stress (Chatterjee et al. 2006; Das et al. 2009; Sarma et al. 2009). LDH, the terminal enzyme of the glycolysis pathway which reversibly converts pyruvate to lactate and also involved in both glycolysis and gluconeogenesis depending on the tissue, is a very good stress indicator, as it shows higher activity in the aerobic tissues like muscle during oxygen tension and in the liver during gluconeogenesis (Verma et al. 2007). In the present study also, the whole-body LDH activity increased significantly with an increase in length of confinement, and significant differences between the two packing densities (40 and 80 g/l) were observed at 24 and 36 h. The observed effects in LDH activity may be due to production of lactate, which indicates oxygen-limited condition in the muscles due to increased restlessness of fish with time or enhanced gluconeogenesis in liver (Verma et al. 2007). Our result is in agreement with the findings of Vijayan et al. (1997) who observed increased hepatic LDH activity in confined tilapias, *Oreochromis mossambicus*.

The whole-body MDH activity, an enzyme of citric acid cycle which is involved in the reversible conversion of L-malate into oxaloacetate, also increased significantly with increase in length of confinement. This increase in the MDH activity with respect to the length of confinement is concomitant with the rise in the AST activity. Increased MDH activity could be to utilize the product/substrate (oxaloacetate) due to the higher activity of AST for production of more energy (ATP), which may be utilized for other physiological activities (Verma et al. 2007). However, unlike other parameters in this study, no significant difference was observed in the activity of this enzyme due to increased packing density (80 g/l).

Glucose production from gluconeogenesis assumes importance during stress under the influence of cortisol. Gluconeogenesis utilizes the non carbohydrate sources for glucose production. When compared to terrestrial animals, fish are better equipped to utilize non carbohydrate sources for energy production. In the present study, activities of gluconeogenic enzymes, G6Pase and FBPase, were found to increase with respect to the increase in the length of confinement and higher packing density, indicating mobilization of non carbohydrate sources as energy resource. Concomitant rise in the whole-body glucose during the experiment could probably be due to higher gluconeogenesis. Similar rise in gluconeogenic enzyme, FBPase, was also observed in brook char *Salvenilus fontinalis* when cultured at higher stocking density (Vijyan et al. 1990).

Amino acids are one of the major substrates for energy in fish (Bever and Dunn 1981). The degradation of amino acids, derived either from the diet or from the breakdown of tissue proteins, occurs first by deamination and then conversion to an intermediate. Energy production through this intermediate takes place either by its entering to the Krebs cycle or by its oxidation. The liver is the main site of amino acid deamination in many fish species

studied so far (Fynn-Aikins et al. 1993; Enes et al. 2006; Kumar 2009). There are, in addition, reports of muscle aminotransferase activity in fishes (Sarma et al. 2009). In the present study, the activities of two transaminases, AST and ALT, increased significantly with the increase in the time period of confinement. This suggests that confinement stress generates higher free amino acid mobilization, which in turn might have produced glucose to cope up with the stress, via the process of higher gluconeogenesis. Higher activity of AST in the higher packing density at 12 h and that of ALT at 12, 24, and 36 h also confirms that higher packing densities can be stressful. Similar observation was recorded in tilapia after being exposed to confinement stress (Vijayan et al. 1997). Sea bass (*Dicentrarchus labrax*) and sea bream (*Sparus aurata*) juveniles raised in semi-intensive conditions had higher transaminases activity when compared to those raised in normal densities (Roncarati et al. 2006).

Adenosine triphosphatase (ATPase) is a membrane-bound enzyme, responsible for the transport of ions through the membrane and immediate release of energy. In the present investigation, the ATPase activity increased with the increase in the length of confinement as well as higher packing density. This indicates that both these stressors caused ion-regulatory dysfunction. However, increased ATPase activity might have hydrolyzed the high-energy phosphate (ATP), and that energy might have been utilized to maintain ionic gradient across the membrane (Das 2002). This result is in tune with the findings of earlier investigation in *Oreochromis mossambicus*, in which confinement stress was shown to increase the intestinal and renal ATPase activity (Quabius et al. 1997; Nolan et al. 1999).

Acetylcholine is a neurotransmitter found in the nerve synapse, and the enzyme AchE breaks the compound to prepare for new nerve conduction. Several studies have demonstrated that AchE enzyme gets inhibited by various toxicants (Assis et al. 2007; da Fonseca et al. 2008) and also by combination of various stressors like pesticide and pathogen (Eder et al. 2007). The inhibition of this enzyme due to immobilization (Gabriel and Soliman 1983) and restraint (Rao and Raju 2000) has also been observed in rats. In agreement with the above studies, the present study result also showed that AchE activity decreased significantly with the increase in the time of confinement. In addition, its activity was significantly lower in the higher packing density at 12 and 24 h, indicating the inhibitory effect of packing density on AchE activity.

Overall, the results suggest that confinement due to packing in small quantity of water is stressful, and metabolic readjustments are required to cope even in the specified optimum density i.e., 40 g biomass/l for 12 h. However, higher packing density and longer duration of transportation further aggravate the stress condition as revealed by higher mortality and increased metabolic activity. Thus, the results of this work validate that the packing density used by hatchery managers is sound from the physiological point of view, besides being economically viable. We also conclude that all enzymes may not serve as equally good markers of stress. FBPase, an enzyme of the gluconeogenesis pathway, and ALT, a transaminase, show higher sensitivity to stress, as the activity levels of these enzymes increased at much higher level when compared to other enzymes due to both the factors under study (higher packing density and increased length of confinement). Further studies on *L. rohita* and other tropical freshwater fishes under different stress conditions are needed to validate this fact.

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