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Secondary stress responses in Indian major carps *Labeo rohita* (Hamilton), *Catla catla* (Hamilton) and *Cirrhinus mrigala* (Hamilton) fry to increasing packing densities

Nirupama Chatterjee, Asim K Pal, Tilak Das, Manush S Mohammed, Kamal Sarma, Gudipati Venkateshwarlu & Subhas C Mukherjee

Physiology Pharmacology and Therapeutic Cell, Central Institute of Fisheries Education, Versova, Mumbai, India

Correspondence: A K Pal, Physiology Pharmacology and Therapeutic Cell, Central Institute of Fisheries Education, Fisheries University Road, Versova, Mumbai 400061, India. E-mail: akpal.53@rediffmail.com

Abstract

Glycogen content and metabolic enzyme activities viz. lactate dehydrogenase (LDH), malate dehydrogenase (MDH), aspartate amino transferase (AST) and alanine amino transferase (ALT) in Indian major carps, *Labeo rohita*, *Catla catla* and *Cirrhinus mrigala*, were investigated after a 6 h transportation trial to compare the species-specific variation and the effect of increased packing density on the metabolism. Fish (45 ± 5 mm, 0.5 ± 0.1 g) were packed in three densities (100, 150 and 200 L^{-1}) for the experiment, and 12 specimens of each species were randomly sampled from all the treatments at the end of transportation. The glycogen content of *L. rohita* ingerlings decreased significantly ($P < 0.05$) with increasing packing density. The activities of enzymes LDH, MDH, AST and ALT showed a rising trend with increasing packing density in all the three species. Species-specific differences were observed in various tested parameters at the lowest packing density (100 fry L^{-1}). Alanine amino transferase and LDH activities were significantly ($P < 0.05$) lower in *C. mrigala* as compared with the other two species. However, glycogen reserves and MDH activity were not significantly different ($P > 0.05$) among the species. The present study reveals that the optimum packing density for Indian major carp fry (100 fry L^{-1}) for transportation up to 6 h and metabolic regimes are species specific during transportation.

Keywords: enzyme activity, glycogen, Indian major carps, metabolism, packing density, stress

Introduction

Indian major carps, *Labeo rohita*, *Catla catla* and *Cirrhinus mrigala* form the mainstay of inland aquaculture in India, which contributed about 87% of the total freshwater production of the country in 1999 (ICLARM 2001). Cultured fish are often classified according to the trophic niche that they occupy in a water body. Of the three species of Indian major carps, *C. catla* occupies the surface layer; *L. rohita* occupies the column layer; and *C. mrigala* occupies the lowest niche in a pond, making them an ideal combination for polyculture (Jhingran 1991). Therefore, it is a common practice to transport the seeds of these three species together for stocking in grow-out ponds.

It is well known that the crowding of fish in a confined space during transportation results in hyperactivity and strain; deterioration of water quality because of the accumulation of harmful gases and wastes; and depletion of oxygen, which often leads to large-scale mortality (Singh, Vartak, Balange & Ghughuskar 2004). In India, hatchery managers determine the packing density on the basis of the size of the fish and the duration of transportation. However, there are no reports available on the effect of increased packing density on the physiology of Indian major carps.

Stress response in all vertebrates including fish results in the activation of the neuro-endocrine system, which brings about changes in metabolism, osmoregulation and haematology (Barton 2000). The metabolism shifts from anabolism to catabolism to supply

the extra energy needed to combat stress (Pickering 1992). Much of the stress studies on fish have been focused on the primary response, i.e. cortisol and catecholamine levels (Barton & Iwama 1991). Of the secondary stress response parameters, blood glucose and lactate levels are the most commonly tested. Besides, glycogen level is another important indicator of secondary stress response. Measurements of metabolic enzyme activity can also serve as valuable stress indicators in organisms like marine invertebrates and fish, where the accurate determination of fields metabolic rates is difficult (Dahlhoff 2004).

Therefore, the present study was designed to find out the metabolic responses to increased packing density in the three species of Indian major carps, and to recommend the optimum packing density of these tested species for transportation, based on their metabolism.

Materials and methods

Experimental procedure

Advanced fry of *L. rohita, C. catla* and *C. mrigala* (45 ± 5 mm, 0.5 ± 0.1 g) were netted from nursery ponds on a farm site and conditioned in hapas (cloth enclosures) under cool showers for 6 h. Fry (one-third of each species approximately) were then introduced together in polythene bags (45×30 cm) containing 1 L water in three densities, i.e. 100, 150 and 200 fry per bag. The air from the bags was squeezed out and medical-grade oxygen was filled in. The bags were then tied tightly with rubber strings. These packets were further placed in cardboard boxes to provide insulation. Each treatment was maintained in duplicate to avoid errors due to packing conditions. The packed fish were transported from the Government fish seed farm, Khopoli, India, to the Central Institute of Fisheries Education, Mumbai, India, by road. The total duration of confinement stress was 6 h. On arrival to the laboratory, six fish of each species were randomly selected from the first bag at each packing density for glycogen estimation and six fish from the second bag at each packing density were used for enzyme assay.

For testing survival (%), another similar experiment was performed under similar conditions with six different replicate bags (at three different packing densities: 100, 150 and 200 fry L^{-1}). At the end of the experiment, dead fish were separated species wise and counted from each bag to assess survival (%).

Sample preparation and estimation of parameters

Fish were anaesthetized with clove oil ($50\ \mu L\ L^{-1}$) and were killed by decapitation, dissolved in 30% KOH and further treated with 95% alcohol to extract glycogen. For enzyme assay, whole-body tissue was homogenized from the remaining fish in chilled 0.25 M sucrose solution using a mechanical tissue homogenizer. The homogenized samples were centrifuged (3000 *g* at 4 °C for 10 min), and the supernatants were collected and stored at -80 °C for subsequent enzyme assays.

Glycogen was estimated using the colorimetric method using anthrone (Hassid & Abraham 1957). Lactate dehydrogenase (LDH) and malate dehydrogenase (MDH) activities were measured by the change in optical density (OD) at 340 nm for 5 min using the method of Wroblewski and Ladue (1951) and Ochoa (1955) respectively. Aspartate amino transferase (AST) and alanine amino transferase (ALT) activities were measured by the estimation of oxaloacetate and pyruvate released, respectively, after incubation of the reaction mixture at 37 °C for 60 min (Wooton 1964). All the enzyme activities were determined using a UV–VIS spectrophotometer (Jasco, Tokyo, Japan). The protein content of the supernatant was analysed using the method of Lowry, Rosebrough, Farr and Randall (1951).

Statistical analyses

Packing density-dependent relation on the tested parameters (Glycogen, LDH, MDH, ALT and AST) of Indian major carps (*C. catla, L. rohita* and *C. mrigala*) and species-wise variation at the lowest packing density were analysed using one-way analysis of variance via statistical software (SPSS, VERSION 11.0). *Post hoc* tests in all cases were carried out using Tukey's multiple comparison procedures, if they were significantly different.

Results

An increase in packing density resulted in an enhanced metabolic rate in all the three species of Indian major carps, as shown in Table 1. The glycogen content of *L. rohita* fry at a packing density of 200 L^{-1} was significantly lower ($P < 0.05$) than the other two treatments. However, the glycogen contents of *C. catla* and *C. mrigala* were not significantly ($P > 0.05$) different among the various treatments. Increased enzyme

Table 1 Effect of increased packing density and species-specific variations in glycogen content, Lactate dehydrogenase (LDH), Malate dehydrogenase (MDH), Aspartate amino transferase (AST) and Alanine amino transferase (ALT) activities and survival (%) of *Catla catla, Labeo rohita* and *Cirrhinus mrigala* fry packed at three different densities of 100, 150 and 200 L^{-1}

Parameters	Packing density (number $^{-}$ ᴸ)	Species variation		
		C. catla	*L. rohita*	*C. mrigala*
LDH	100	258.36 ± 14.65^{A}	279.17 ± 8.69^{aA}	46.00 ± 6.56^{aB}
	150	268.95 ± 17.22	353.72 ± 5.14^{b}	93.91 ± 9.31^{b}
	200	282.48 ± 12.04	369.05 ± 12.29^{b}	124.56 ± 11.86^{b}
MDH	100	212.22 ± 6.12^{a}	227.48 ± 30.81^{a}	192.5 ± 27.1
	150	268.95 ± 17.22^{b}	356.72 ± 24.8^{b}	237.66 ± 27.34
	200	285.04 ± 12.94^{b}	369.05 ± 12.29^{b}	264.66 ± 24.4
AST	100	28.06 ± 2.3^{A}	21.80 ± 0.96^{aB}	25.42 ± 0.99^{aA}
	150	29.32 ± 2.29	26.71 ± 1.76^{ab}	29.68 ± 1.43^{a}
	200	33.53 ± 5.14	32.40 ± 3.47^{b}	41.48 ± 4.06^{b}
ALT	100	5.76 ± 2.35^{aA}	8.04 ± 0.3^{B}	2.06 ± 0.12^{aC}
	50	6.99 ± 0.26^{ab}	10.51 ± 0.94	2.42 ± 0.25^{ab}
	200	7.84 ± 0.52^{b}	10.8 ± 1.16	2.94 ± 0.15^{b}
Glycogen	100	15.23 ± 1.14	14.07 ± 1.54^{a}	17.99 ± 1.26
	150	12.00 ± 0.40	13.11 ± 0.89^{a}	16.10 ± 1.81
	200	12.26 ± 1.85	9.69 ± 1.22^{b}	13.96 ± 0.69
Survival (%)	100	94 ± 3^{a}	92 ± 4^{a}	94 ± 5^{a}
	150	68 ± 6^{a}	62 ± 2^{a}	$75 \pm 7a$
	200	61 ± 6^{b}	56 ± 5^{b}	72 ± 5^{b}

LDH and MDH activity is expressed as Δ0.01 optical density mg protein $^{-1}$ min $^{-1}$ at 25 °C. Aspartate amino transferase activity is expressed as nanomoles oxaloacetate released mg protein $^{-1}$ minute $^{-1}$ at 37 °C. AST activity is expressed as nanomoles pyruvate released mg protein $^{-1}$ min $^{-1}$ at 37 °C. ALT activity is expressed as nanomoles pyruvate released mg protein $^{-1}$ min $^{-1}$ at 37° C. Glycogen is expressed as mg g $^{-1}$ wet tissue.

Different superscripts (a, b, c) in the same column indicate a significant difference among different packing densities in each species. Different superscripts (A, B, C) in the same row indicate a significant difference among the species at the lowest packing density ($P < 0.05$) (Tukey's multiple range test, $\alpha = 0.05$). Values are expressed as mean ± SE ($n = 6$).

activity of all tested enzymes with increasing packing density was evident in all the three species. Lactate dehydrogenase activity was significantly less ($P < 0.05$) at the lowest packing density, i.e. 100 fry L^{-1} as compared to the other two densities in *L. rohita* and in *C. mrigala*. A similar trend was observed for MDH activity in *L. rohita* and *C. catla*. Transaminase enzyme AST was significantly higher ($P < 0.05$) at the highest packing density in *L. rohita* and *C. mrigala*. Similarly, a significantly higher ALT ($P < 0.05$) was evident at the highest packing density in *C. catla* and *L. rohita*.

Species-specific difference was tested at the lowest packing density (100 fry L^{-1}) as this packing density was found to be optimum for transportation. Alanine amino transferase and LDH activities were significantly ($P < 0.05$) lower in *C. mrigala* as compared to the other two species. However, glycogen reserves and MDH activity were not significantly different ($P > 0.05$) among the species.

Survival (%) decreased with increasing packing densities in all the three species tested ($P < 0.05$) (Table 1).

Discussion

Transportation of Indian major carps for stocking is mainly carried out in the fry and fingerling stages. As this size is small and does not yield adequate amount of tissue sample, we have measured glycogen content and enzyme activity from whole fish bodies. Although this procedure does not give tissue-specific glycogen content and enzyme activity, it is considered to be a useful procedure, when working with small-sized fish. A similar procedure was followed and found to be suitable for the estimation of glycogen, glucose and lactate from small-sized inland and anadromous stripped bass *Morone saxatilis* exposed to handling stress (Reubush & Heath 1996).

Biochemical parameters serve as reliable indicators of the physiological status of organisms (Ferry-Graham & Gibb 2001). Stress response in fish includes a cascade of reactions such as primary, secondary and tertiary responses. It is initiated after the perception of stress in the hypothalamus, which in turn activates the pituitary–inter-renal axis, result-

ing in the release of the hormones cortisol and catecholamine. The secondary stress response includes metabolic, haematological and immunological changes because of the action of cortisol and catecholamine. The tertiary response is the final stage when the fish is no longer able to sustain the stress, which leads to disease or exhaustion and finally death. Catecholamine and cortisol induce glycogenolysis and gluconeogenesis respectively. Both processes together cause a rise in the blood glucose level. Blood glucose and hepatic glycogen are therefore commonly measured parameters of stress response (Manush, Pal, Das & Mukherjee 2005). In the present study, the glycogen content of *L. rohita* decreased significantly with increasing packing densities. Glycogen is broken down into glucose in the early stages of stress (Mazeaud, Mazeaud & Donaldson 1977; Barton & Iwama 1991). the present result is consistent with previous reports of glycogen depletion in tilapia after 1 h of transportation (Orji 1998) and *Heteropneustes fossilis*, after being exposed to increased loading density (Shrivastava & Sahay 1987). It is reported that the depletion of hepatic glycogen in tilapia confined for 2 h, which resumed after 24 h of confinement, indicates that glycogen reserves in tilapia are utilized only in the initial stages of stress (Vijayan, Pereira, Grau & Iwama 1997). However, in our study, the glycogen levels did not differ significantly in the other two species between the different packing densities, which indicates species-specific adaptation to increasing confinement stress. Similar reports are available in common dentex (*Dentex dentex*) exposed to handling stress; glycogen reserves were not affected (Morales, Cardenete, Abellán & García-Rejón 2005).

Metabolism is a physiological process reflecting the energy expenditure of living organisms. As lactate dehydrogenase helps in ATP production in the muscle under anaerobic condition by converting pyruvate into lactate, LDH assay serves as a useful stress indicator. In tissues undergoing gluconeogenesis, the enzyme catalyses the reverse reaction. In the present study, the increase in LDH activity can be attributed to the production of preferred substrate (lactate) for gluconeogenesis (Suarez & Mommsen 1987; Moon & Foster 1995) in the liver. However, a more realistic picture of the mechanism would be clear only if tissue-specific data become available. Similar results were reported in tilapia after being exposed to confinement stress for 20 h (Vijayan *et al.* 1997).

Besides lactate, amino acids are the preferred substrates for gluconeogenesis in fish. Fish utilize protein and lipid sources rather than carbohydrate for energy fulfillment (Demeal 1978). Teleosts are known for their ability to convert amino acids into glucose. Therefore, the higher activity of AST and ALT indicates the mobilization of aspartate and alanine via gluconeogenesis for glucose production to cope with stress. Similarly, tilapia subjected to confinement stress also exhibited a rise in AST activity (Vijayan *et al.* 1997). A concomitant rise in MDH activity along with a rise in transaminases activity is probably because of the role of MDH in the conversion of oxaloacetate, the byproduct of aspartate transamination to pyruvate in the cytoplasm. Pyruvate is further utilized as a substrate for gluconeogenesis.

Species-specific variation in glycogen utilization and enzyme activities is suggestive of their difference in basal metabolism. Higher glycogen content and lower activities of LDH and ALT in *C. mrigala* as observed in the present study emphasize the fact that *C. mrigala* is more tolerant to confinement stress as compared with *L. rohita* and *C. catla*, which may be because of their preferential ecological niche (bottom dwelling). Similar results were evident from our earlier reports in *L. rohita* and *Cyprinus carpio* early fingerlings (Chatterjee, Pal, Manush, Das & Mukherjee 2004). Survival (%) is found to be affected by confinement stress because of increasing packing densities of Indian major carp fry stages. Although from an economic point of view, higher packing densities are considered beneficial, our *prima-facie* biochemical evidence indicates the need to optimize the packing densities in Indian major carps.

The overall results of the present investigation suggest that packing densities have a marked effect on the metabolism in Indian major carps, and crowding and confinement stress exhibits a species-specific variation. Therefore, it is vital to optimize the packing density depending on the duration of transportation; size of fish, species and metabolic responses can potentially be used as biochemical markers. From our study, it is evident that a packing density of 100 fry L^{-1}, which approximately corresponds to 50 g biomass L^{-1}, is considered to be optimum for transportation for a period of 6 h.

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