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Metabolic Elasticity and Induction of Heat Shock Protein 70 in *Labeo rohita* Acclimated to Three Temperatures

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ABSTRACT : The metabolic response of *Labeo rohita* to thermal acclimation was assessed. Advanced fingerlings of *L. rohita* (average weight 31±1.4 g) were acclimated to 31, 33 and 36°C compared with ambient temperatures (26°C) for 30 days and different enzymes associated with stress response were estimated. Glycolytic enzyme-Lactate dehydrogenase, (LDH, E.C.1.1.1.27), TCA cycle enzyme-Malate dehydrogenase (MDH, E.C.1.1.1.37), Protein metabolizing enzymes-Aspartate amino transferase (AST, E.C.2.6.1.1) and Alanine amino transferase (ALT, E.C.2.6.1.2) of liver, gill and muscle, Gluconeogenic enzymes-Fructose 1,6 Bi phosphatase (FBPase, E.C. 3.1.3.11) and Glucose 6 phosphatase (G6Pase, E.C. 3.1.3.9) of liver and kidney were significantly ($p<0.05$) different with increasing acclimation temperatures. Heat Shock Protein-70 (HSP-70) was expressed in increasing intensity at 31, 33 and 36°C but was not expressed at 26°C. Results suggest that higher acclimation temperatures enhance metabolism and *L. rohita* maintains homeostasis between 26-36°C *via* an acclimation episode. Such adaptation appears to be facilitated by resorting to gluconeogenic and glycogenolytic pathways for energy mobilization and induction of HSPs. (**Key Words :** Thermal Acclimation, *Labeo rohita*, Metabolic Activities, Heat Shock Protein 70)

INTRODUCTION

Temperature is one of the important abiotic factors, which influences biochemical reactions and therefore has a significant impact on the physiology and biochemistry of ectothermic organisms. Increase in temperature, up to optimum level, favors aquaculture by reducing the time required to produce marketable size animals and producing more number of generations per year. On the contrary, temperature adversely affects the health of aquatic animal by increasing metabolic rates, assisting proliferation, invasiveness and virulence of bacteria and other pathogens that cause a variety of pathophysiological disturbances in the host (Wedemeyer et al., 1999; Hayford et al., 2002). Higher temperature acclimation in different fishes viz., *Rhinomugil corsula* (Kutty, 1981) and *Salvelinus alpinus* L (Baroudy and Elliott, 1994) is accompanied by changes in the relative activities of glycolytic and mitocohondrial enzymes, that is a compensatory decrease of oxidative enzymes and constant activities of glycolytic enzymes (Guderley, 1990). Thermal acclimation process influences the tolerance limit and oxygen consumption (Das et al., 2004) and their limits have been determined by enzyme activities in different temperature regimes (Kita et al., 1996). On the other hand, high protein (Sudarman and Ito, 2000) and other nutraceuticals are being used to counter stress due to thermal stress (Zulkifli et al., 2004; Chung et al., 2005).

Exposure of cells or whole organisms to heat shock results in a reversible increase in the synthesis of some acute phase proteins against subsequent shock, known as heat shock proteins (Iwama et al., 1998, 1999; Currie et al., 2000; Palmisano et al., 2000; Ming et al., 2003), which play an important role in maintaining homeostasis. The expression of heat shock proteins (HSP) has been demonstrated in many organisms in response to a variety of stressors and environmental contaminants other than thermal stress (Sanders, 1993; Forsyth et al., 1997; Feder and Hofmann, 1999). The most abundant and widely studied group of stress proteins is the HSP70-protein family,

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Table 1. Composition of experimental diet (% DM basic)

Ingredients	(g/100 g feed)
Soybean meal	45.1
Fishmeal	18.3
Wheat flour	14.6
Corn flour	15.0
Cotton seed meal	3.0
Cod liver oil	1.5
Carboxy methyl cellulose	1.0
Vitamin-Mineral mix[1]	0.5
Vitamin C	0.95
Vitamin B complex[2]	0.05
Proximate composition	
Moisture	90.23
Crude protein	35.67
Ether extract	5.21
Nitrogen free extract	49.35
Ash	9.77
Energy*	386.97

[1] Composition of vitamin-mineral mix (Agrimin) Agrovet India Pvt. Ltd. (quantity kg^{-1}), Vitamin A, 625,000 IU; Vitamin D_3, 62,500 IU; Vitamin E, 250 mg; Nicotinamide, 1 g; Cu, 312 mg; Co, 45 mg; Mg, 6 g; Fe, 1.5 g; Zn, 2.13 g; I, 156 mg; Se, 10 mg; Mn, 1.2 g; Ca, 247.34 g; P, 114.68 g; S, 12.2 g; Na, 5.8 mg; K, 48.05 mg.

[2] Composition of vitamin B complex, (Becosules) Glaxo-Smilthkline Pvt. Ltd. (quantity g^{-1}), Thiamine mononitrate, 20 mg; Riboflavin, 20 mg; Pyridoxine hydrochloride, 6 mg; Vitamin B_{12}, 30 mcg; Niaciamide, 200 mg; Ca pantothenate, 100 mg; Folic acid, 3 mg; Biotin, 200 mcg.

* Energy was calculated as digestible energy, DE (kcal/100 g) = (CP%×4)+(EE%×9)+(TC%×4)

comprising constitutive as well as inducible isoforms and is highly conserved across phyla from bacteria to mammals (Schlesinger et al., 1982). It is the most commonly induced stress protein in response to sub-optimal physiological conditions (Hutchinson et al., 1994). Cellular functions of HSP70 proteins include the stabilization of unfolded protein precursors before assembly, translocation of proteins into organelles, rearrangement of protein oligomers, dissolution of protein aggregates and refolding or degradation of denatured proteins (Feige et al., 1996). Heat shock protein studies in fishes are still in the early stages as compared to those in bacteria, yeast and mammals.

Labeo rohita is one of the widely cultured Indian major carps, which is a preferred fish through out the India, owing to its high commercial value in domestic market. Although there is a preliminary report on the induction of HSP70 in *Cirrhinus mrigala* in response to heat stress is available (Paromita et al., 2005), but there are no investigation being carried out in *L. rohita.* Knowledge of the acclimatory response of *L. rohita* to thermal stress is expected to provide a better know-how on their culture potential in various agro-climatic regions of India. The present study was intended to investigate metabolic response and expression of heat shock protein (HSP70) of *L. rohita* acclimated to three temperatures.

MATERIALS AND METHODS

Experimental fishes and acclimation procedure

Fingerlings of *L. rohita* of average weight (mean±SE: 31±1.4 g) were brought from Khopoli fish seed farm, Government of Maharashtra, to Central Institute of Fisheries Education, Mumbai and were held in the laboratory conditions (26±1°C) for 30 days. Each of the fishes was deprived of food for 24 h prior to the experiment.

Acclimation of fishes (6/aquarium) to 31, 33 and 36°C i.e., Δ5, Δ7 and Δ10 over average ambient temperature (26°C) were carried out in a thermostatic aquarium (52 liters water capacity, sensitivity±0.2°C) at 1°C per day to reach the test temperatures as per the method described (Beitinger et al., 2000) and maintained them for another 30 days. Our previous investigations of thermal tolerance in Indian Major Carps (Das et al., 2004); *Cyprinus carpio* (Chatterjee et al., 2004) and in *Macrobrachium rosenbergii* (Manush et al., 2004) suggested that the fishes or shellfishes are completely acclimated to test temperatures in 30 days. Similar acclimation studies in other species, sheepshead minnow, *Cyprinodon variegatus* (Bennet and Beitinger, 1997) were considered as the reference for acclimation. A fixed photoperiod of 12L:12D (Light:Dark) was maintained with light exposure from 6.00 h to 18.00 h. Round the clock aeration was provided in all the experimental containers to maintain the dissolved oxygen level. Other water quality parameters; pH, ammonia-N, nitrite-N and nitrate-N were monitored at every 5 days interval and maintained at the optimum rearing conditions for *L. rohita* (Das et al., 2005) and were fed to satiation with pelleted feed (35% crude protein) prior to acclimation and during experimental acclimation period as recommended for *L. rohita* (Renukardhyay and Varghese, 1986). The proximate composition of the experimental feeds is represented in Table 1. Feeding was done twice a day (8.00 h and 20.00 h) till the end of 30 days. Siphoning of waste feed and faecal materials were done each day before dispensing the feed. Water exchange was carried out up to 25% of water with fresh chlorine free water every day.

Chemicals

All the chemicals were procured from E-Merck, Germany and Himedia Laboratories, Mumbai, India unless otherwise mentioned.

Metabolic studies

Sample preparation : Acclimated fishes were anaesthetized with clove oil (50 µl/L) and were immediately killed by decapitation. Vital organs; liver (0.3 g), gills (0.7 g), kidney (0.1 g) and muscle (0.8 g) were dissected out and were homogenized in chilled sucrose solution (0.25 M) by mechanical tissue homogenizer and centrifuged (5,000 rpm at 4°C for 10 minutes). Supernatant

Table 2. Effect of acclimation temperatures on enzyme parameters (AST, ALT, LDH, MDH, G6Pase and F1,6BPase of *L. rohita* acclimated to 26, 31, 33 and 36°C)

Parameters	Acclimation temperatures (°C)			
	26	31	33	36
AST				
Liver	2.57±0.21[a]	4.14±0.21[b]	4.30±0.65[b]	4.51±0.24[b]
Gill	2.28±0.24[a]	3.97±0.44[ab]	4.55±0.55[b]	5.41±0.5[b]
Muscle	10.02±0.57	10.03±0.55	10.22±1.04	11.96±1.27
ALT				
Liver	3.80±0.41[a]	3.94±0.20[ab]	4.35±0.22[ab]	5.76±0.76[b]
Gill	2.76±0.32[a]	3.55±0.40[ab]	3.77±0.22[ab]	5.07±0.66[b]
Muscle	13.54±0.28[a]	14.03±1.34[ab]	14.06±0.74[ab]	16.79±0.42[b]
LDH				
Liver	70.70±15.24[a]	94.16±3.6[ab]	118.56±9.65[b]	128.7±1.9[b]
Gill	183.87±28.76	237.57±31.37	118.56±21.9	275.6±20.2
Muscle	224.6±42.3[a]	431.8±20[b]	437.8±21.8[b]	443.5±37.9[b]
MDH				
Liver	238.78±13.5[a]	274.0±4.4[ab]	321.6±16.4[bc]	358.6±14.9[c]
Gill	170.0±8.4[a]	193.5±17.3[ab]	256.6±10.2[bc]	271.5±23.5[c]
Muscle	148.6±34[a]	263.47±10.39[b]	282.9±4.9[b]	280.0±42.5[b]
G6Pase				
Liver	0.16±0.007[a]	0.21±0.02[ab]	0.24±0.01[bc]	0.29±0.002[c]
Kidney	0.24±0.007[a]	0.32±0.01[a]	0.42±0.01[b]	0.47±0.03[b]
F1,6BPase				
Liver	0.21±0.01	0.29±0.05	0.34±0.03	0.39±0.06
Kidney	0.14±0.009[a]	0.17±0.02[a]	0.21±0.01[ab]	0.27±0.03[b]

AST was expressed as nano moles of oxaloacetate formed per mg protein per minute at 37°C. ALT was expressed as nano moles of pyruvate formed per mg protein per minute at 37°C. LDH and MDH were expressed as specific activity per mg protein per minute at 37°C. G-6-Pase and FBPase were expressed as microgram phosphorus released per mg protein per minute at 37°C. Different superscripts (a, b, c) in the same row indicate significant difference amongst different acclimation temperatures in each organ ($p<0.05$) (Turkey's multiple range test, $\alpha = 0.05$). Values are expressed as mean±SE (n = 6).

were collected and preserved frozen condition (-80°C) for subsequent enzyme analysis.

Enzyme assay

Activities of ALT and AST (Wotton, 1964), LDH (Wrobleuiski and Ladue, 1955), MDH (Ochoa, 1955) were determined from liver, muscle and gill tissues. Similarly, F16BPase (Freeland and Harper, 1959) and G6Pase (Marjoric, 1964) were determined from liver and kidney tissues. Supernatant was analyzed for total protein content (Lowry et al., 1951). All the enzyme activities were determined with a UV-VIS spectrophotometer (Jasco, Japan).

HSP70 analysis

HSP70 resolved by SDS-PAGE and immunodetected after western blotting (Towbin et al., 1979). Liver tissue (20%, w/v) was homogenized in Tris buffer (pH 7.5) under chilled condition and with protease inhibitor 0.1 mM PMSF (phenyl methane sulfonyl fluoride). The homogenate (n = 6) were centrifuged at 5,000 rpm to remove large particulate matter and the supernatant was analyzed for total protein content (Lowry et al., 1951). Sample buffer was immediately added to each sample and then heated to 95°C for 2 min. Sub samples of uniform protein (50 μg) were separated by SDS-PAGE with 12% separating and 5% stacking polyacryalmide gels (Blatter et al., 1972) using an electrode buffer (Laemmli, 1970). Hela cell lysate (heat shocked, Bioreagents-LYC 101 F, Stressgen Canada) (20 μg) was loaded to one lane to serve as an internal standard for blotting efficiency. Proteins were separated at 1.5 mA per well for approximately 3 hours and then electro blotted on to a total PVDF (Polyvinylidene fluoride) transfer membrane (E578-10×10 cmSQ, USA) at 200 mA for 3 hours. After blotting, gels were stained with Coomassie blue to ensure that complete transfer had occurred. Membranes were blocked with 3% Bovine Serum Albumin and Tris Buffer Saline (pH 7.4). Tween 20 (0.05%) in Tris Buffer Saline was used as washing solution. Primary monoclonal antibodies HSP70 developed against carps (1:1,000 dilution, Bioreagents-SPA 810, Stressgen, Canada) were used as probes. Since these detect only inducible forms of HSP70, HSC-73 was not detected in our studies. Horseradish peroxidase-conjugated goat anti mouse IgG (1:2,000 dilution, GENEI, Bangalore) was used to detect HSP70 probes. Bound antibodies were visualized by Gel

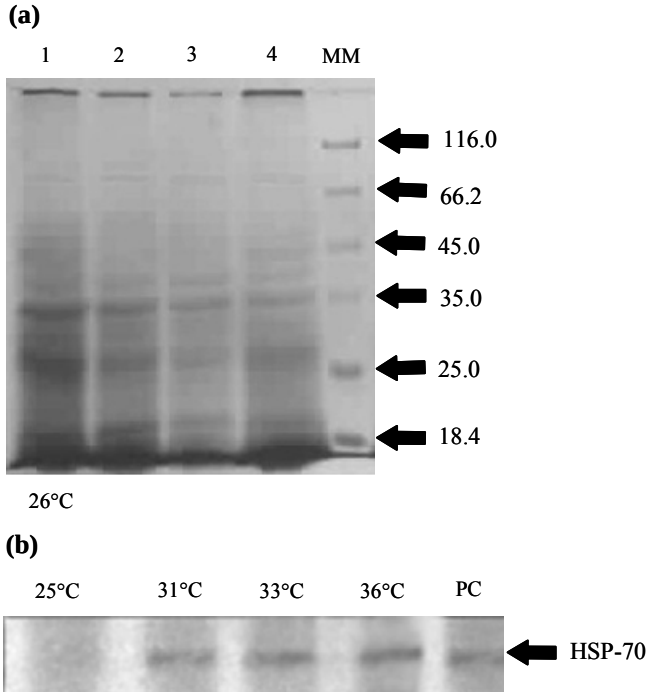

Figure 1. Photographs of (a) 12% SDS polyacylamide gel showing persistence of protein profiles of *Labeo rohita* acclimated to 31, 33 and 36°C. Proteins of heat stressed fishes were resolved in Lanes 1, 2, 3, 4 and 5 (a). Lane 1-26°C (ambient), Lane 2-31°C, Lane 3-33°C, Lane 4-36°C. Lane (MM)- Molecular weight marker. Arrow in the right margin indcates the proteins with respective molecular weights. (b) Western blot showing persisitence of HSP70 at higher temperatures (31, 33, 36°C and positive control) in Lane 2, 3, 4 and 5 respectively.

Documentation system (Syngene, UK) and were quantified using densitometry.

Statistical analysis

Statistical significance of enzyme activities and HSP70 was analyzed using one-way analysis of variance (ANOVA *via* the computer statistical package, SPSS 11.0 for Windows). Tukeys's multiple range test was carried out for post hoc comparison of means ($p<0.05$), if they were significantly different.

RESULTS

Metabolic responses of thermal acclimation

Data pertaining to the enzyme activities; G6Pase and FBPase in liver and kidney at different acclimation temperatures (31, 33 and 31°C) over ambient temperature (26°C) are presented in Table 2. Results of G6Pase activity in liver and kidney tissue and FBPase activity in kidney were significantly ($p<0.05$) different. Data pertaining to the response of different acclimation temperatures on enzyme activities of LDH, MDH, ALT and AST in liver gill and muscle of *L. rohita* are also presented in Table 2. LDH activity differs significantly ($p<0.05$) in liver and muscle tissue. However, the values were not significant ($p>0.05$) in gills. MDH activity was significantly ($p<0.05$) different in liver, gill and muscle (between 26 and 36°C). ALT activity showed significant ($p<0.05$) difference in all test organs between 26 and 36°C. AST activity showed a significant ($p<0.05$) increase in liver and gills. However, the values were not significantly different ($p>0.05$) in muscle at all acclimation temperatures.

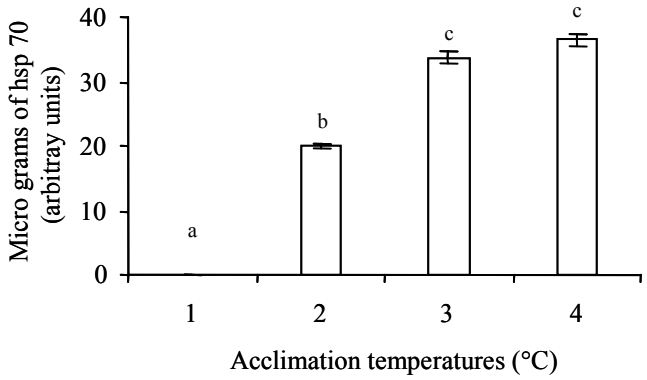

Figure 2. HSP70 proteins in liver tissue of *L. rohita* are shown densitometer readings of protein bands were detected by western blotting. (Acclimation temperatures: 1-26°C, 2-31°C, 3-33°C and 4-36°C. Different superscripts (a, b, c) in the same row indicate significant difference amongst different acclimation temperatures ($p<0.05$) (Turkey's multiple range test, $\alpha = 0.05$). Values are expressed as mean±SE (n = 6).

Enzyme activities were tissue specific in the present investigation. ALT, AST and LDH activity were highest recorded in muscle tissue, MDH and F1, 6Bpase activity were highest in liver and G6Pase was highest in kidney tissues. However, enzyme activities demonstrated a common increasing trend with increasing temperatures.

Acclimatory response and expression of HSP70

Acclimation to higher temperatures (31, 33 and 36°C) resulted in an increased level of inducible HSP-70 expression in liver of *Labeo rohita*. However, HSP70 was not detectable at 26°C (Figures 1 and 2).

DISCUSSION

The main aim of the study was to assess metabolic responses of *L. rohita* in response to thermal acclimation. High temperature creates higher free amino acid mobilization, as higher enzyme activities of ALT (Alanine Amino Transferase) and AST (Aspartate Amino Transferase) were observed in *L. rohita*. ALT and AST enzyme activities were increased in tune with increasing acclimation temperature, which in turn produces glucose to

cope up with the stress, in the process of higher gluconeogenesis. Similar observation was recorded in Tilapia after being exposed to confinement stress (Vijayan et al., 1997).

LDH activity showed a significant increase at higher temperature (36°C). It may be due to the higher production of lactate, which is the preferred substrate for gluconeogenesis in fish (Suarez and Mommsen, 1987; Moon and Foster, 1995). Present study suggests that the hepatic capacity of lactate mobilization is more due to higher metabolic condition or in stressed fish (Vijayan et al., 1997). Absolute value of LDH activity was higher in muscle, which may be due to muscle glycolysis (Milligan and Girard, 1993).

Malate Dehydrogenase (MDH), is an enzyme of TCA cycle, was increased at higher acclimation temperature in order to use the product (oxaloacetate) due to the higher activity of AST for production of more energy (ATP), which may be utilized for other physiological activities. In the present study, there was significant increase in MDH activity in fishes acclimated at higher temperatures, which strengthens the above hypothesis. Maximum glucose (data unpublished) mobilized was through non-carbohydrate source; mainly by protein, as ALT and AST activities were more at higher temperatures. Results also strengthen the fact that higher acclimation temperature induces amino acid mobilization (Alanine and Aspartate) in *L. rohita*.

Glucose-6-phosphatase (G-6Pase) is an enzyme, which catalyzes the conversion of glucose-6-phosphate to glucose. In the present study, G-6Pase was found to have significantly increased at higher acclimation temperatures, which indicates higher glycogen mobilization for blood glucose production (Vijayan et al., 1990). Fructose 1-6 bi phosphatase (a gluconeogenic enzyme) activity was increased at higher acclimation temperatures, which indicates that the higher temperature increases the gluconeogenic activity in *Labeo rohita*. Similar observation was made in Brook Charr in response to stress due to confinement (Vijayan et al., 1990).

Acclimation is the process by which long-term exposure to a novel environmental condition results in remodeling of cellular machinery to adapt to the new environment. Acclimation to higher temperatures results in higher basal levels of HSP70 (Dietz and Somero, 1992). In the present investigation, HSP70 was expressed at 31°C, 33°C and 36°C in increasing intensities. However, HSP70 was not expressed at 26°C (Figures 1 and 2). Primary antibody (SPA 810) precisely estimates only inducible HSP70 and not their constitutive forms (HSC 73). Thus in normal cells, inducible HSP70 are not being expressed but constitutive forms may be present.

HSP70 plays important role in protein biogenesis under normal cellular conditions, which is significant during the growth of organisms. Thus enhanced protein synthesis requires increased amount of HSPs (Pal and Mukherjee, 2003). In natural conditions, expression of heat shock proteins in fish varies with season (less during winter than in summer). Maximum accumulation of HSP70 was recorded in the digestive gland and gills of mussels (*Mytilus galloprovincialis*) during summer period (Mineir et al., 2000). So it can be considered as an acclimatization or adaptive mechanism of the animal to the changing environment. In our study, increased induction of HSP was recorded in *L. rohita* with increasing temperatures (26 to 36°C), by which they adapt to the acclimatory procedure. It is interesting to note that there is no significant difference in induction of HSP70 between 33 and 36°C (Figure 1), which indicates that the test fishes are able to adapt to higher test temperatures (33 and 36°C) if gradually acclimated. A similar trend in the metabolic responses at 33 and 36°C strengthens our hypothesis (Table 1). However, our study couldn't describe the induction of HSP expression due to cold acclimation as the acclimation temperatures selected were above ambient conditions (26°C).

Sub lethal thermal stress may lead to increased amount of HSP induction and cross protection against subsequent lethal stressors. There can be a positive correlation between ability to induce stress proteins and survival. Medaka (*Orizias latipes*) embryos were unable to induce stress protein synthesis in earlier stages and were more susceptible to heat shock than embryos at later stages where HSP induction was observed (Werner et al., 2001). One of our previous study showed that thermal tolerance range was increased with increasing acclimation temperatures in *L. rohita* (Das et al., 2004), which may be due to the increasing levels of heat shock proteins. In the present study, it was observed higher levels of HSP at higher temperatures. Hence, it could be concluded that HSP plays key role to protect the organisms from unfavorable condition and maintain their homeostasis. Therefore, our future research would be concentrating on the beneficial use of heat shock proteins in cross protection in abating lethal stressors.

The present study indicates that higher acclimation temperature induces gluconeogenesis and glycogenolysis in *L. rohita*. The energy level was maintained by gluconeogenesis, using lactate and amino acids as a substrate. HSP70 induction serves as a protective mechanism in combating stress at higher acclimation temperatures. Overall results suggest that *L. rohita* can be well adapted to the three test temperatures (31, 33 and 36°C) by gradual acclimation procedure.

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