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Effect of low temperature on metabolic enzymes and HSP-70 expression of coldwater fish *Barilius bendelisis*

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Abstract

The levels of key metabolic enzymes viz. Glucose 6 phosphate dehydrogenase, Lactate dehydrogenase, Pyruvate kinase, Acetylcholine esterase and ATPase were monitored at varying time intervals in coldwater fish *Barilius bendelisis* exposed to 5°C. The levels of these enzymes increased to 236.23, 28.22, 68.66, 83.59 and 143.55% respectively as compared to control group of fishes during 96hrs of rearing in cold. The enzymatic values of pyruvate kinase, lactate dehydrogenase and ATPase levels increased considerably ($P < 0.01$) just after 24 hrs of treatment while glucose 6 phosphate dehydrogenase and esterase increased significantly ($P < 0.01$) after 48 hrs of cold treatment. Isozyme profiling of above enzymes also supports the data visually. Moreover, two more isoforms of Pyruvate kinase were also observed in cold adapted fish specimens than in control group. Twelve protein bands were also visualized when the samples from test fishes were run on the SDS-PAGE, one band of 29kD at Rf of 0.63 exhibited relatively high intensity in the fish reared at cold temperature as compared to control group. Similarly, elevated levels of Hsp-70 were also detected ($p < 0.05$) due to the cold stress given to the fishes for a period of 24- 96 hrs that also served as an early indicator of temperature stress in fish and played an important role in cold adaptation.

Introduction

Temperature, being the most important environmental factors, influences the normal physiological process of fish. Hill stream fishes living in cold climate appear to adapt a compensatory strategy that sustains levels of metabolism and performance more or less constant despite changes in environment temperatures (5-25°C) (D'Amico et al.

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2002). Under acute thermal stress, fish will rely on number of adaptations that are under neural and endocrine control to ensure that they meet the energy requirement needed for their physiological compensation and acclimation to changing environment (Kuo and Hsieh 2006). The physiological responses include the consumption of metabolic energy, which is essential for maintaining body functions and regulation. Enzyme function, in particular, is often extremely sensitive to temperature change and this sensitivity is probably related to the balance maintained between flexibility and stability in discrete, relatively mobile regions of the polypeptides involved in catalysis. The resultant marginal stability is usually assumed to be a necessary attribute of enzyme (Zavobszky et al. 1998; Fields et al. 2002). Muscle ATPase, lactate dehydrogenase (LDH), acetylcholine esterase (AChE), glucose 6 phosphate dehydrogenase (G6PDH), Pyruvate Kinase (PK) are the key enzymes of glycolysis, pentose phosphate pathway and muscle contraction etc. and involved in various metabolic activities in cell. Since activity alteration of these metabolic enzymes is highly related to the production and utilization of energy, the integrated coordination of these enzyme activities implies a status of stress responses and change in activities may facilitate clarification of these metabolic pathways and energy utilization in fish under cold climate. On the other hand expression of heat shock proteins (HSP) / molecular cheperone under stressful environmental conditions is another interesting area, to understand mechanism behind cold tolerance in fishes as reported by Ju et al. (2002) and Weber et al. (2005), who found the abundance of heat shock proteins-70 (HSP-70) rapidly increased in brain and muscle tissues of channel cat fish in response to cold acclimation.

Coldwater fisheries, a component of the inland fisheries, are very important from the point of view of tourism and sports besides being a source of cheap animal protein. Though contribution of coldwater fisheries to inland sector in India is meager but it has the vast natural resources to make a dent and contribute to the economy of the hilly regions. Among coldwater fishes, *Barilius spp.* are one of the most dominant species in the streams, rivers and lakes all along the Himalayas. They are found in snow fed fast flowing water of hill streams, though some are found in the big rivers in locations with a gravel or sandy substrate. *Barilius* are found across Asia. They are most common in India, but also occur in Afghanistan, Bangladesh, Nepal, Myanmar, Pakistan, Sri Lanka and Thailand. They have developed a unique character to resist cold climate (below 10°C). Hence, it was chosen as a representative species under present investigation to get insight in to the mechanism of cold tolerance at biochemical level in coldwater fishes of Indian subcontinent.

Materials and Methods

Test fish, *Barilius bendelisis* weighing 15.7 - 30.0 g were netted out from local streams of Kumaon Himalayas' using cast nets and live fish were transported to wet lab

in polyethylene bags filled 1/3 with water and inflated with oxygen. Fish were kept in flow through tubs for 15 days to remove transportation stress and adapting them to confined conditions.

Experiments were designed in glass aquaria (length 46.0 cm, width 30.0 cm, height 30.0 cm) of 15-liter capacity. Clean aquaria were filled with fresh water up to desired level (12 liters) and aerated to maintain optimum dissolved oxygen level. Four glass aquaria fitted with proper aeration were installed at 5°C of water temperature in cold cabinets and one-glass aquarium was maintained at 20°C of temperature as control using thermostats. Next morning, seven live specimens of fish *B. bendelisis* were released in each glass aquarium after slowly mixing the water in the aquarium so that the thermal shock to fish could be avoided. Fish samples were taken out at regular intervals of 24 hrs, 48 hrs, 72 hrs and 96 hrs from the four aquaria kept at 5°C. Fish kept as control and maintained at 20°C were also collected after 72 hrs.

The tissues were collected aseptically from the skeletal muscles by making an incision on either side of the dorsal fin. Tissues were kept in pre-autoclaved 1.5 ml eppendorf tubes and sealed with parafilm immediately before freezing at -20°C for further use. Frozen tissue samples (0.2-0.8 g) were thawed at 4°C and protein was extracted by homogenization for 10 minutes (homogenization was carried out manually with a glass head rod and a glass homogenizing tube) using approximately 1.5 - 2.0 ml of 0.9% saline. Homogenized samples were then centrifuged at 5000 rpm for 10 minutes at 4°C to remove the debris. The supernatant was used for various biochemical analyses.

Enzyme activity of glucose-6-phosphate dehydrogenase (G6PDH; E.C.1.1.1.49) was measured by the protocol of [Kuo and Hsieh \(2006\)](#) using 3 mM glucose-6-phosphate as substrate in 100 mM Tris buffer (pH 8.0) having 0.25 mM MgCl₂ and 0.6 mM NADP at 30°C. Lactate dehydrogenase (LDH; E.C.1.1.1.27) activity was measured using the protocol of [Bergmeyer and Bernt \(1974\)](#) using a mixture of 2.4 mM sodium pyruvate and 0.1 mM NADH₂ as substrates dissolved in 100 mM sodium phosphate buffer (pH 7.5). Activities were estimated at 14°C by decreasing absorbance at 340 nm of NADH₂. Pyruvate kinase (PK; E.C.2.7.1.40) was measured by using protocol of [Edwards et al \(1984\)](#). The reaction solution having 100 mM Tris HCl buffer (pH 7.4), contained 165 mM KCl, 5.5 mM MgCl₂, 0.1 mM NADH₂, 0.75 mM ADP, 5.5 mM DTT and 0.66 mM phosphoenolpyruvate (PEP). Activities were assayed at 14°C and the absorbance was measured at 340nm. The activity of acetylcholine esterase (AChE; E.C.3.1.1.1) was assayed by the method described by [Wolfgang et al. \(1974\)](#) in 0.1 M sodium phosphate buffer (pH 7.5) using 1.65 mM of acetylcholine as substrate. Activities were assayed at 37°C by taking absorbance of colour developed at 490 nm.

The ATPase (EC 3.6.1.3) activity was measured in purified myosin ([Martone et](#)

al .1986) using 2 ml reaction mixture containing 100 l NaCl (2.5 M), 800µl tris buffer (50 mM pH-7.4), 100µl CaCl₂ (100 mM), 5µl BME, 5µl ATP (0.1 M) as a substrate and 75µl of enzyme extract. The reaction was stopped after 30 min of incubation at 30°C with 1.5 ml of 20 % TCA and colour was developed by the method of Tausky and Shorr (1953).

One unit of enzyme in above assays is that amount of protein, which produces either 1nm of product or consumed 1nm of substrate/minute/mg of protein.

Soluble protein was quantified using the method of Lowry et al. (1951) with bovine serum albumin as a standard. The visualization of isoenzymes on gels was carried out by the methods described by Pasteur et al. (1988) with certain modifications using specific substrates of enzymes with NBT (1%) and PMS (1%) to form formazon precipitates in case of oxido-reductase enzymes (G6PDH, LDH and PK). Similarly, the esterases were stained on gel using /β -Naphthyl ester and Fast blue RR to form colored complex. The procedure of Laemmli (1970) was used to check the protein-banding pattern in skeletal muscles of fishes and to detect variations due to cold stress. The levels of HSP-70 were estimated in fish muscles by ELISA using the protocol of Peridigon et al. (1991). The goat polyclonal antibody raised against carp HSP-70 as primary antibody were obtained from Santa Cruz Biotechnology Inc California. Anti-goat rabbit antibody as secondary antibody conjugated with peroxidase used in ELISA was supplied by Genei (India)

Data are presented as mean + SEM (n=10). Student's t-test was used to compare the significance of differences in mean values between groups.

Results

Behavioural changes

The fish maintained in aquaria at 20°C were observed to be more active and swam rapidly as compared to those at 5°C. They became very sluggish when transferred to 5°C and subsequently settled down at the bottom with reduced opercular movements within 30 minutes after the onset of the experiment.

Variations in enzyme levels

The activities of enzymes related to energy metabolism, i.e., glucose 6-phosphate dehydrogenase (G6PDH), lactate dehydrogenase (LDH), pyruvate kinase (PK), acetylcholine esterase (AChE) and myosin ATPase were monitored and presented in table 1.

Table 1. Impact of cold on key metabolic enzymes of *Barilius bendelisis*

| Time (Hrs) | Enzyme activities (U*min ⁻¹ *mg ⁻¹) | | | | |
|--------------------|---|-----------------------------|---------------------------|----------------------------|--------------------------|
| | G6PDH | LDH | PK | AchE | ATPase |
| Control (20°C) | 8.28 ± 2.43 | 2905.18 ± 158.9 | 83.12 ± 4.4 | 118.14 ± 11.48 | 24.8 ± 4.76 |
| 24 | 8.48 ± 1.95 (2.42) | 3095.54 ± 184.4* (6.55) | 93.41 ± 6.8* (12.38) | 127.71 ± 30.91 (8.10) | 30.4±2.53* (22.58) |
| 48 | 11.51 ± 1.45* (39.0) | 3305.42 ± 324.4* (13.78) | 111.85 ± 8.7* (34.56) | 148.32 ± 12.07* (25.55) | 43.4 ± 3.49* (75.0) |
| 72 | 13.46 ± 2.09* (62.56) | 3485.32 ± 402.3* (19.97) | 135.23 ± 13.1* (62.69) | 175.21 ± 31.81* (48.30) | 56.5 ± 3.38* (127.82) |
| 96 | 27.84± 3.63* (236.23) | 3725.19 ± 241.6* (28.23) | 140.19 ± 12.6* (68.66) | 216.9 ± 10.36* (83.59) | 60.4 ± 7.22* (143.55) |

Values are mean ± SEM for 10 numbers of observations

* Mean values are significantly higher ($P \leq 0.01$)

Values in parenthesis are % increase in enzyme activity as compared to control

The glucose 6-phosphate dehydrogenase activity increased by 2.42 %, 39.0 %, 62.56 % and 236.23 % at 24, 48, 72 and 96 hours of cold exposure at 5°C as compared to a control maintained at 20°C for 72 hours. The activity of this enzyme was found to be 27.84 3.63 U*min⁻¹ *mg⁻¹ after 96 hrs. in the cold exposed fish. The activities of the enzyme were observed to be significantly higher ($P \leq 0.01$) in *Barilius bendelisis* samples reared for 48, 72 and 96 hours respectively at 5°C than the control set.

Similarly, lactate dehydrogenase (LDH) activity showed an increase of 1.07, 1.14, 1.19 fold and 1.28 folds when cold exposure was given for 24, 48, 72 and 96 hours respectively in comparison to the control set. The increase was statistically significant ($P \leq 0.01$) in all the above sets. Another important glycolytic enzyme, pyruvate kinase also demonstrated a marked increase ($P \leq 0.01$) in its activity with the values being 93.41± 6.8, 111.85±8.7, 135.23±13.1 and 140.19 ±12.6 U*min⁻¹ *mg⁻¹ of protein among all time points after cold treatment respectively than control set reared at 20°C (83.12±4.4 U).

Acetylcholine esterase (AchE) of *Barilius bendelisis* also showed a marked increase ($P \leq 0.01$) in their activities during cold exposure (increase by 8.10 %, 25.54 %, 48.3 % and 83.59 % at 24, 48, 72 and 96 hrs. respectively compared to control set in AchE). However, the increase was not significant ($P \leq 0.01$) at 24 hrs.

Similarly, activity of myosin ATPase increased significantly ($P \leq 0.01$) by 22.58, 75.0, 127.82, and 143.55 % at 24, 48, 72 and 96 hrs. of cold exposure at 5°C respectively as compared to control at 20°C. Moreover, statistically significant increased values ($P \leq 0.01$) of ATPase were observed as the duration of rearing of the fish was increased from 24 hrs. to 96 hrs. But the ATPase activity was not significantly different ($P \leq 0.01$) between the fishes reared at 72 and 96 hrs. in cold.

Visualization of enzymes/proteins on Native PAGE and SDS-PAGE

The physical visualization of enzyme activities of the four enzymes except ATPase was done on Native PAGE using 7.5 % polyacrylamide gel to check appearance of their different isoforms on exposure to cold temperature. Though single band of G6PDH appeared on PAGE gel irrespective of the fish rearing temperature but its intensity appeared to be increasing visually in case of fish muscle samples reared at 5°C for 96 hrs. than the control. This substantiates 236.23% increased level measured by quantitative enzyme assay. Similarly, single band of LDH was also detected on gel but not much variations in the intensity of the bands was observed on physical visualization of PAGE gels though 28.22 % higher activity of LDH was recorded quantitatively in test fishes exposed to cold temperature for 96 hrs. than control. It appears that increase in the activity of the LDH enzyme is not that sufficient to be detected on Native PAGE with naked eyes. On the other hand, Native PAGE of pyruvate kinase enzyme yielded some interesting observations (Figure 1A) with the appearance of two bands at Rf 0.025 and 0.05 in the muscle sample extracts (lane 6-10) of *Barilius bendelisis* reared under cold temperature of 5°C. This signifies the formation of additional isoforms of pyruvate kinase due to cold stress. Increased intensity of AchE (Figure 1B) on gel in cold exposed fish samples also supports our quantitative data of 83.59% increase than control fish reared at 20°C. However, an additional band at Rf 0.55 appeared in some of the lanes of AchE enzyme irrespective of temperature treatment to fish.

The SDS-PAGE was also performed to record the soluble proteins pattern from muscle tissues in order to determine the appearance of new type of protein bands due to cold exposure of fish (Figure 1 C). A gradient gel (10-15% polyacrylamide) using SDS (2%) along with β -mercaptoethanol (2%) was found suitable to resolve denatured proteins optimally. Twelve bands were visible which were of varied intensities but one band at Rf 0.63 having a molecular weight of 29 Kd exhibited relatively high intensity in the fish reared at cold temperature as compared to control (Fig.1 C).

Variations in HSP-70

The final objective was the estimation of Hsp- 70 using previously standardized ELISA. On giving cold stress to fish, increased levels of HSP- 70 were observed by 30.29, 33.41, 60.3, and 55.53 % respectively at 24, 48, 72 and 96 hrs (Fig.2).

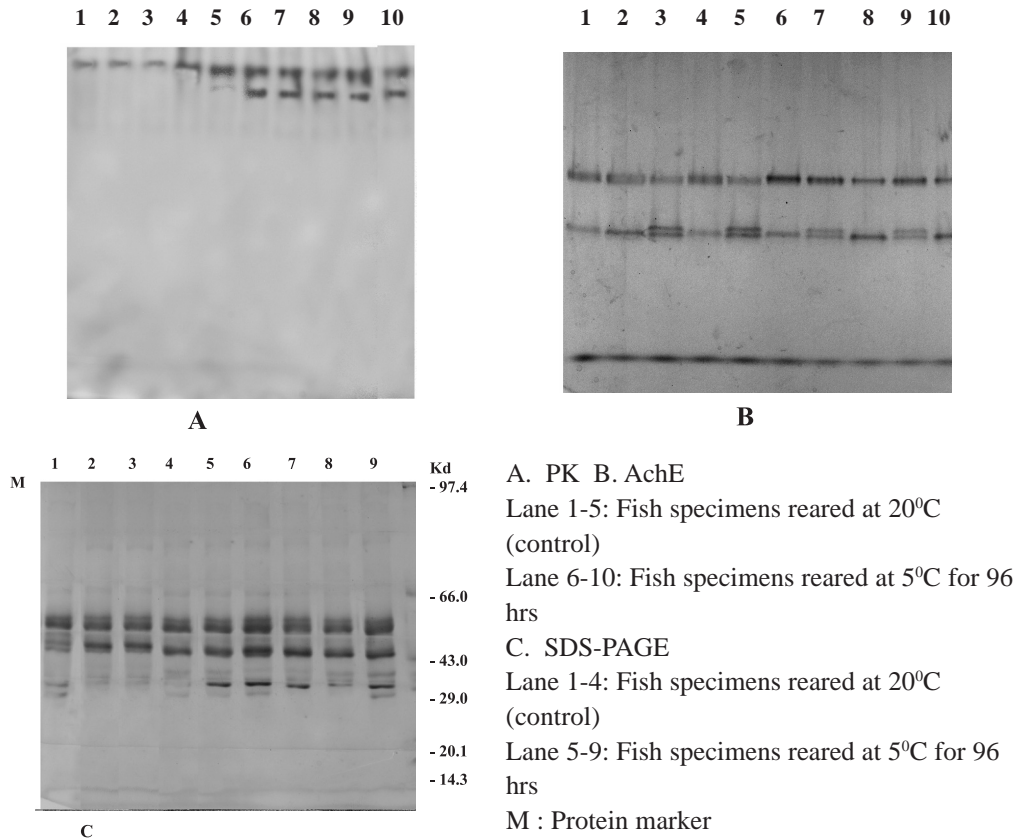
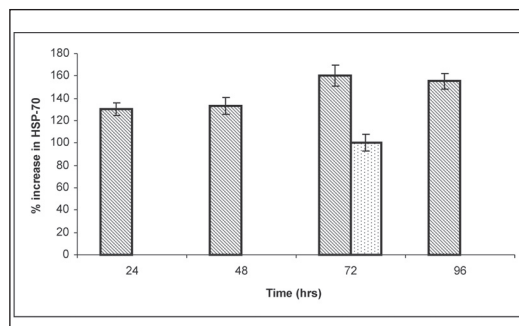


Figure 1: Impact of cold on enzymes/denatured protein profiles on PAGE

Statistical analysis of the data revealed a significant ($P \leq 0.05$) percentage increase in Hsp-70 at 24 hrs. of rearing of fish in cold as compared to control. This increase was further enhanced ($P \leq 0.01$) on increasing the duration of rearing the fish in cold climate



upto 72 hrs. Thus it appears from the data that the expression of Hsp- 70 reaches to a maximum level on 72 hrs. of rearing the fish in cold because no further significant statistical increase ($P \leq 0.05$) was found in the sets of fish reared for 72 and 96 hrs .

Figure 2. Impact of cold on HSP-70 expression of muscle proteins

▨ Fish reared at 5°C for different time intervals ▤ Fish reared at 20°C as control

Discussion

The behavioral changes exhibited by *B. bendelisis* on transfer to low temperature are in consonance with the observations made on *Clarias batrachus* and *Schizothorax plagiostomus* by Joshi et al. (1980) and Bhatt and Singh (1985) respectively. Similarly reduced movements of fins and operculum were also reported previously by Kapila et al. (2002) following sudden transfer of *S. richardsonii* to lower temperatures.

Our results exhibiting increased levels of G6PDH are also in agreement with a recent research done by Kuo and Hsieh (2006) on grass carp, which is recognized as cold tolerant eurythermal cyprinid. Under present investigations the activities of G6PDH in the *Barilius bendelisis* increased steadily on similar pattern to grass carp after 48hrs of cold shock treatment. This indicates that gluconeogenesis was activated in the cold tolerant species under cold shock. G6PDH is a key regulatory enzyme of pentose phosphate pathway that helps in the production of metabolite, which helps in gluconeogenesis. Contrarily, milkfish, a tropical species that is less tolerant to the cold, maintained G6PDH at a stable but at low levels even after cold shock treatment of 48hrs or more (Kuo and Hsieh 2006). Further, Gracey et al. (2004) reported that 252 genes involving 18 genes of metabolism were up regulated during cold in common carp. Moreover, they observed activation of pentose phosphate pathway genes including G6PDH based upon transcript profile of carp liver. These observations further support our finding of upward production of G6PDH indirectly by up regulation at gene level, which supply additional NADPH by pentose phosphate pathway for elevated lipid metabolism in the cold and induces the cold-responsive gene of acyl-CoA 9-desaturase, for membrane adaptation.

In case of LDH, observations of Fields (2001) support our data while working on an Antarctic fish *Chaenocephalus aceratus*. They observed that the LDH orthologs from more cold-adapted species have higher values of catalytic rate constant, or turnover number (k_{cat}), through lower Arrhenius activation energies (E_a), and increased in the apparent Michaelis constant for the substrate pyruvate. Thus they catalyzed the conversion of pyruvate to lactate faster than those from more warm-adapted species. Podrabsky and Somero (2004) further provided evidence for increased LDH activity to cold temperature. Fields and Houseman (2004) observed increased activity of LDH in cold-water fishes by adaptation of enzyme through one to few amino acid substitutions during the process of adaptation. Thus increased levels of LDH in cold adapted fishes may be attributed to molecular flexibility generated by geometrical modifications in structure of enzyme needed to maintain an appropriate catalytic rate and stability of active site for substrate recognition during adaptation.

Hochachka and Somero (2002) reported the effect of temperature on catalytic

and regulatory functions of pyruvate kinase of the rainbow trout and the Antarctic fish *Trematomus bernacchii* and observed K_m value of PK for phosphoenol pyruvate is temperature dependent. Pyruvate kinase is a regulatory enzyme of glycolytic pathway and involved in energy generation. Podrabsky and Somero (2004) support our data with the observations that activities of pyruvate kinase correlate positively with decreasing habitat temperature of *Austrofundulus limnaeus*. On the other hand, Gracey et al. (2004) reported that few genes in brain, gill and kidney, showed increased expression of most glycolytic genes, whereas skeletal muscle showed a decrease using microarray technique. Further Cossins et al. (2006) opined that in fast-twitch skeletal muscle, glycolysis is responsible for supplying most of the ATP needed for contraction, so the activation or repression of glycolytic genes in this tissue may be linked to the expression of the genes that comprise the contractile apparatus, which were found to decrease with cooling. Moreover, Granner and Pilkis (1990) reported PK in rainbow trout occurs in at least two forms. White muscle possesses an isoenzyme, which termed as M-PK, was activated by Fructose 1, 6 diphosphate (FDP) during gluconeogenesis while the second isoenzyme found in liver and brain termed L-PK, was insensitive to FDP. It plays a key role in hepatic glucose and lipid metabolism. Above findings also support our observations of visualizing two isoforms of PK in muscle tissues as both the gluconeogenesis and lipid metabolic pathways are activated in lipid metabolism during cold exposure. Our data of LDH and PK is also in agreement with D'Amico et al. (2002) who reported that increase in activities in glycolytic enzymes (LDH and PK) during cold rearing of fish and suggested further that fish muscles require more amount of energy in the form of ATP for muscle contraction.

Harper et al. (1989) investigated the effect of temperature in the range from 5-35 °C in skeletal muscle from both warm-acclimated and cold-acclimated freshwater teleost fish (*Cyprinus carpio*) and found that cholinesterase activity does not contribute to the differential temperature sensitivity in fish acclimated to high and low temperatures. On the other hand, Wood et al. (1999) also reported that low temperatures may reduce the activity of acetyl cholinesterase as a result relatively increased amount of acetylcholine may interact with the cholinergic receptors on type II cells for maintaining the action potential which is quite contrary to our findings on *Barilius bendelisis*. Moreover, presence of additional band of esterase at an Rf of 0.55 in few fish samples irrespective of the temperature conditions under present investigation appeared to relate with genetic variations in AchE than to any physiological adjustments (Kapila and Mishra, 2006).

ATPase activity of myofibrils from cold adapted species in fast twitch muscles of fish was considerably higher (approx. 2.8 times) at low temperatures than for tropical species (Johnston et al. 1975). Similar increased ATPase activity with cold-acclimation occur in other cyprinids such as common carp (*Cyprinus carpio*) and roach, *Rutilus*

rutilus (Heap et al. 1985) are on same lines with our observations with *Barilius bendelisis*. On the other hand Walesby and Johnston (1981) reported that the net effect of increased levels of ATPase is not a widespread phenomenon among teleosts. Moreover, Wakeling et al. (2000) suggested that the increase in the ATPase activity was dependable on cold exposure duration for teleost fish because the maximum cruising speed of some freshwater fish is increased at low temperatures and decreased at high temperatures after several weeks of cold acclimation. In the trout heart, acclimation to cold increases the activity of myosin-ATPase (Aho and Vornanen 2000) due to formation of new type of myofibrils having isoforms of myosin heavy chain.

Increase in the level of Hsp-70 proteins due to heat shock has been studied by many workers and reviewed extensively by Basu et al. (2002) and Iwama et al. (1999) but little work has been carried out on expression of these proteins due to cold exposure in fish. Up regulation of Hsp-70 in mammalian skeletal muscle is suggested to play an important role in preventing muscle damage or atrophy in response to environmental stressors (Oishi et al. 2003). Similarly, Currie et al. (2000) suggested that the particular family of Hsp must be considered when evaluating the stress by molecular means as they visualized that increased Hsp-70 levels are specific indicators of early detection of stress due to thermal acclimation in fish. Our findings are in agreement with the observations of Ju et al. (2002) who found rapidly increased levels of Hsp-70 in the brain tissue of channel catfish in response to cold acclimation. Similarly, Weber et al. (2005) working on same lines found the abundance of Hsp-70 mRNA in the fish reared at cold temperature on days 14 and 28 of the study. Our data also supports the observations of Laios et al. (1997) where increased expression of Hsp-70 in rat cardiomyocytes and in the muscle of carp during cold shock was detected.

In conclusion, exposure of cold temperature of 5°C to *Barilius bendelisis* brings out adjustments in the levels of key metabolic enzymes/proteins by altering their amounts (G6PDH, AchE, PK, ATPase, HSP-70), synthesizing new isoforms (PK) and changing their kinetic properties by shifting geometry of active site so that energy can be generated for various physiological activities required for survival of fish in order to combat cold.

References

- Aho, E. and M. Vornanen. 2000. Cold-acclimation increases basal heart rate but decreases its thermal tolerance in rainbow trout (*Oncorhynchus mykiss*). *Journal of Comparative Physiology* 171: 173-179.
- Basu, N., A.E. Todgham, P. A. Ackerman, M.R Bibeau, K. Nakano, P.M. Schulte and G. K. Iwama. 2002. Heat shock Proteins genes and their functional significance in fish. *Gene* 295: 173-183.
- Bermeyer, H.U. and E. Bernt. 1974. *Methods of enzymatic analysis*. 2nd edition. Academic press, New York, 1307 pp.
- Bhatt, S.N. and H. R. Singh. 1985. Effect of thermal variation on some haematological values of *Schizothorax plagiostomus* (Heckel). *Uttar Pradesh Journal of Zoology* 5: 2004-2009.
- Cossins, A., J. Fraser, M. Hughes and A. Gracey. 2006. Post-genomic approaches to understanding the mechanisms

- of environmentally induced phenotypic plasticity. *Journal of Experimental Biology* 209: 2328-2336.
- Currie, S., C.D. Moyes and B.L. Tufts. 2000. The effects of heat shock and acclimation temperature on heat shock protein 70 and heat shock protein 30 mRNA expression in rainbow trout: in vivo and in vitro comparisons. *Journal of Fish Biology* 56: 398-408.
- D'Amico, S., P. Claverie, T. Collins, D. Georgette, E. Gratia, A. Hoyoux, M. A. Meuwis, G. Feller and C. Gerday. 2002. Molecular basis of cold adaptation. *Transactions of Royal Society of London* 357: 917–925
- Edwards R.J., C.H. Rodeck and D.C. Watts. 1984. Pyruvate kinase in fetal plasma and amniotic fluid unsuccessful for the prenatal diagnosis of Duchenne muscular dystrophy. *American Journal Medical Genetics* 18:231-235.
- Fields, P. A. 2001. Protein function at thermal extremes: balancing stability and flexibility. *Comparative Biochemistry and Physiology* 129: 417–431.
- Fields, P. A. and D. E. Houseman .2004. Decreases in Activation Energy and Substrate Affinity in Cold-Adapted A_4 -Lactate Dehydrogenase: Evidence from the Antarctic Notothenioid Fish *Chaenocephalus aceratus*. *Molecular Biology and Evolution* 21: 2246-2255.
- Fields, P. A., Y. S. Kim, J. F. Carpenter and G. N. Somero. 2002. Temperature adaptation in *Gillichthys* (Teleost: Gobiidae) A_4 -lactate dehydrogenase: identical primary structures produce subtly different conformations. *Journal of Experimental Biology* 205:1293–1303.
- Gracey, A.Y., E. J. Fraser, W. Li, Y. Fang, R. R. Taylor, J. Rogers, A. Brass and A.R. Cossins .2004. Coping with cold: An integrative, multitissue analysis of the transcriptome of a poikilothermic vertebrate. *Proceedings of National Academy of Science (USA)* 101: 16970–16975.
- Granner, D. and S. Pilgis . 1990. The isozymes of Pyruvate kinase in zebra fish. *Journal of Biological Chemistry* 265: 10173–10176
- Harper A. A., J. R. Shelton and P. W. Watt .1989. The temperature dependence of the time course of growth and decay of miniature end-plate currents in carp extraocular muscle following thermal acclimation. *Journal of Experimental Biology* 147: 237-248
- Heap, S. P., P. W. Watt, and G. Goldspink .1985. Consequences of thermal change on the myofibrillar ATPase of five freshwater teleosts. *Journal of Fish Biology* 26: 733-738.
- Hochachka, P. W. and G. N. Somero .2002. Biochemical adaptation: mechanism and process in physiological evolution. Oxford University Press, Oxford 208 pp.
- Iwama, G. K., M. M. Vijayan, R. B. Forsyth and P. A. Ackerman .1999. Heat shock proteins and physiological stress in fish. *American Journal of Zoology* 39: 901 -909.
- Johnston, I. A., W. Davison and G. Goldspink .1975. Adaptations in Mg^{2+} -activated Myofibrillar ATPase induced by temperature acclimation. *Federation of European Biological Society Letters* 50: 293-295.
- Joshi, B.D., L.D. Chaturvedi and R. Debral .1980. Some haematological values of *Clarius batrachus* L. following its sudden transfer to varying temperatures. *Indian Journal Experimental Biology* 18 (1): 76-77.
- Ju, Z., R.A. Dunham, and Z. Liu .2002. Differential gene expression in the brain of channel of cat fish (*Ictarus punctatus*) in response to cold acclimation. *Molecular Genetics and Genomics* 268 : 87-95.
- Kapila, R., S. Kapila and Y. Basade .2002. Impact of temperature variation on haematology and serum enzymes of *Schizothorax richardsonii*. *Indian Journal of Fisheries* 49 (2): 187-192
- Kapila, R and D.P. Mishra .2006. Eterase as a marker for identification of genetic variations in coldwater fish, *Schizothorax richardsonii* (Gray). *Journal of .Inland Fisheries Society of India* 38(1):68-71.
- Kuo, C.M. and S.L. Hsieh .2006. Comparisons of physiological and biochemical responses between milk fish and grass carp to cold shock. *Aquaculture* 251 : 525-536
- Laios, E., I. M. Rebeyka, and C.A. Prody .1997. Characterization of cold – induced HSP expression in neonatal rat cardiomyocytes. *Molecular Cellular Endocrinology* 173 : 153 – 159.
- Laemmli, U.K .1970. Cleavage of structure proteins during the assembly of the head of bacteriophages T4. *Nature* 227: 680-685.
- Lowry O.H., N.J. Rosenbrough, A.L. Farr and R.J. Randall .1951. Protein measurement with the folin phenol reagent . *Journal of Biological Chemistry* 193 : 267-275.

- Martone, C.B., L. Busconi, E.J. Folco, R.E. Trucco and J.J. Sánchez, .1986. A simplified myosin preparation. from marine fish species. *Journal of Food Science* 51:1554-1555.
- Oishi, Y., K. Taniguchi, F. Kawano, A. Ishihara and Y. Ohira . 2003. Up regulation of HSP-70 in reloading rat soleus muscle after prolonged hindlimb unloading. *Japanese Journal of Physiology* 53 : 281-286.
- Pasteur, N., G. Pasteur, F. Bonhomme, J. Catalan, and J.B.Davidson .1988. Staining protocols. In. *Practical isozyme genetics*, pp 95-150. Ellis Horwood Ltd, Chichester.
- Perdigon, G., M.E.N. De Macias, S. Alvarez, G. Savoy de Giori, M. Medici, and M. Nunez de Kairuz .1991. Behaviour of natural and heated yoghurt in the immune system and preventive capacity on enteric infection. *Milchwissenschaft* 46: 411-416.
- Podrabsky, J.E. and G.N. Somero .2004. Changes in gene expression associated with acclimation to constant temperatures and fluctuating daily temperatures in an annual killifish *Austrofundulus limnaeus*. *Journal of Experimental Biology* 207: 2237–2254.
- Taussky, H.H. and E. Shorr . 1953. Enzymatic assay of actomyosin ATPase assay. *Journal of Biological Chemistry* 202: 675-685.
- Wakeling, J.M., N.J. Cole, K.M. Kemp and I.A. Johnston. 2000. The biomechanics and evolutionary significance of thermal acclimation in the common carp *Cyprinus carpio*. *American Journal of Physiology* 279: 657–665
- Walesby, N.J. and I. A. Johnston .1981. Temperature acclimation of $Mg^{2+}Ca^{2+}$ myofibrillar ATPase from a cold-selective teleost, *Salvelinus fontinalis*: A compromise solution. *Experientia* 37 : 116- 718.
- Weber, T.E., B.C. Small and B.G. Bosworth .2005. Lipopolysaccharide regulates myostatin and MyoD independently of an increase in plasma cortisol in channel cat fish (*Ictalurus punctatus*). *Domestic Animal Endocrinology* 28: 64 – 73.
- Wolfong B. F., G. Steiner and C. Kuhne .1974. Enzymatic synthesis of acetylcholine by a serotonin containing neuron from helix. *Nature* 252: 631-637.
- Wood, P. G., O. V. Lopatko, S. Orgeig, J. R. Codd, and C. B. Daniels .1999. Control of pulmonary surfactant secretion from type II pneumocytes isolated from the lizard, *Pogona vitticeps*. *American Journal of Physiology* 277: R1705 -R1711.
- Zavodszky, P., J. Kardos, A. Svingor, and G. A. Petsko .1998. Adjustment of conformational flexibility is a key event in the thermal adaptation of proteins. *Proceedings of National Academy of Science (U. S. A.)* 95: 7406-7411