

Glycerol accumulation and modulation in selected enzyme activities in response to cold acclimation of two species of cyprinid fishes *Schizothorax richardsonii* (Gray) and *Barilius bendelisis* (Hamilton)

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Abstract

Glycerol accumulation in blood plasma and modulation in the activities of selected enzymes associated with glycerol synthesis in the liver under cold temperature were assessed in two freshwater cyprinid fishes, *Schizothorax richardsonii* (snow trout) and *Barilius bendelisis* (Indian hill trout). Fishes were collected from Kosi River, Uttarakhand during late October 2011 (water temperature 15-16 °C) and after an acclimatisation for 7 days they were maintained at lower temperature of 5 °C over a period of 96 h. The decrease in temperature triggered higher accumulation of glycerol in the blood plasma of both the species. Glycerol accumulation in plasma was found to be associated with changes in enzyme in the liver. In both the species, liver exhibited higher activities of glycerol-3-phosphate dehydrogenase (GPDH) and higher concentration of dihydroxy acetone phosphate (DHAP) which gets converted into glycerol. The increased activities of different enzymes involved in amino acid metabolism indicated the utilisation of amino acids as carbon source in glycerol synthesis. The measure of enzymes associated with gluconeogenesis indicated typical gluconeogenic pathway associated with glycerol synthesis. Based on the enzyme profile studied, it was established that the freshwater cyprinids; *S. richardsonii* and *B. bendelisis*, a decrease in water temperature triggered the release of glycerol in the blood plasma and also activated metabolic response for glycerol accumulation. The higher accumulation of glycerol seems to contribute towards cold adaptation in these species.

Keywords: Gluconeogenesis, Glycerol, Glycerol-3-phosphate, Glycerol-3-phosphate dehydrogenase, Glycolysis, Indian hill trout, Snow trout

Introduction

Schizothorax richardsonii and *Barilius bendelisis* are common cold water fishes found in the snow fed hill streams and lakes of Himalayan regions of India. They are short distance migratory species and such migration is mainly performed for food and spawning (Sharma, 1989; Sahoo *et al.*, 2009). *S. richardsonii*, commonly called as snow trout, is widely distributed in the Himalayan streams at altitudes ranging from 1400 to 3000 m above mean sea level (Sivakumar, 2008). *B. bendelisis*, commonly called Indian hill trout, is also found at altitudes ranging from 600 to 2700 m above mean sea level (Negi and Nautiyal, 2002). These species play significant role in the capture fishery in several parts of the Himalayan region and are a demanding ornamental as well as potential food fish. During winter, they can sustain temperatures as low as 5-6 °C and in summer they thrive at 20-22 °C. Decrease in water temperature may result in a number

of physiological and behavioural consequences in fishes. As temperature reaches the lower thermal tolerance limit of a species, a number of cascading physiological and behavioural responses occur. The magnitude and duration of temperature change and the acclimation of individuals for such alteration can influence the extent of the consequences of cold response in fishes. The sublethal effects of cold acclimation can be studied in terms of cold response in fishes (Tanck *et al.*, 2000; Van den Burg *et al.*, 2005). The mechanism of cold tolerance in fishes has been thoroughly studied and different enzymatic processes responsible for low temperature adaptation were also investigated in many fish species (French *et al.*, 1981; O'Gardy, 1982; Davies *et al.*, 1988; Raymond, 1992, 1995; Raymond *et al.*, 1996; Driedzic *et al.*, 1998; Raymond and Hassel, 2000; Ewart *et al.*, 2001; Fletcher *et al.*, 2001; Lewis *et al.*, 2004; Driedzic *et al.*, 2006). At low temperature, rainbow smelt (*Osmerus mordax*) accumulates very high concentrations of glycerol which

acts as antifreeze agent (Raymond, 1992; Clow *et al.*, 2008). It was also reported that the level of glycerol and amino acid metabolising enzymes are several fold higher in the liver of rainbow smelt than in other teleosts during winter (Driedzic *et al.*, 1998). Cold acclimation seems to be a complex process involving different enzymes associated with carbohydrate metabolism and amino acid metabolism involved in glycolysis and gluconeogenesis, which play a significant role in adjusting the physiological processes in extreme cold condition (Driedzic *et al.*, 1998; Treberg *et al.*, 2002a; Kapila *et al.*, 2009).

In cold adapted fishes, the decrease in temperature acts as an external stimulus for the onset of different metabolic processes leading to higher accumulation of glycerol and other enzymatic activities which is inevitable for their survival (Treberg *et al.*, 2002b). The source of glycerol production is liver glycogen and the carbon sources for glycerol appear to be stored glycogen and amino acids which are accessible through the gluconeogenic pathways (Raymond, 1995; Raymond and Driedzic, 1997). Furthermore, the higher levels of enzymes involved in amino acid metabolism play critical role in amino acid utilisation in glycerol production (Driedzic *et al.*, 1998). It was further established that glycerol appears to be synthesised by conversion of dihydroxyacetone phosphate (DHAP) to glycerol-3-phosphate (G3P), a reaction catalysed by glycerol-3-phosphate dehydrogenase (GPDH). Most of these studies were carried out in rainbow smelt (*Osmerus mordax*), capelin (*Mallotus villosus*), Atlantic tomcod (*Microgadus tomcod*) and flounder (*Liopsetta putamani*) which are marine species. The present study was undertaken to test similar phenomenon working in the freshwater cyprinids *Schizothorax richardsonii* and *Barilius bendelisis* for cold acclimation process through glycerol accumulation in blood plasma. We also tried to investigate different enzyme activities associated with glycerol production during cold acclimation.

Materials and methods

Fish samples and experimental design

Specimens of *S. richardsonii* and *B. bendelisis* were caught in late October 2011 using cast net from river Kosi, near Almora, Uttarakhand. Fishes (25 nos.) were transported to the wet laboratory of Directorate of Coldwater Fisheries Research at Bhimtal, Nainital and held in indoor flow-through FRP tanks. Fishes were allowed to acclimatise to their respective temperature under proper aeration. The ambient water temperature at the time was approximately 15 °C. The fishes were fed on formulated diet (Joshi, 2004) daily during the acclimatisation period (7 days) and also during the experiment. Apart from

sporadic incidental mortalities, experimental fish appeared to be in good condition throughout the experiment.

Fishes were maintained in two experimental groups (12 in each group) in glass aquaria of 20 l capacity: one aquarium was maintained at approximately 5-6 °C by adding ice flakes and the other one at ambient temperature (15-16 °C) and monitored regularly with the help of mercury thermometer. Initial weight of the *S. richardsonii* and *B. bendelisis* were in the range of 60 ± 5.2 g and 59 ± 4.1 g respectively. The live specimens of both species were released in each aquarium after slowly mixing the water in the aquarium to avoid any thermal shock. Three specimens of each species from both tanks were sampled at regular intervals of 24, 48, 72 and 96 h. Samples were also collected at the time of stocking (0 h; served as control) in the tank. The experiment was performed in triplicate to minimise the error.

Sample collection and preparation

Fishes were killed by a blow to the head and blood was drawn from caudal vein using a 1ml heparinised syringe and collected in EDTA coated tubes. The blood samples were centrifuged at 5000 g for 10 min at 4 °C and the plasma samples were transferred to 1 ml eppendorf tubes and stored at -20 °C for future analysis. Liver samples were quickly removed and used immediately for enzyme assays, while muscle samples were stored at -20 °C for future analysis.

Biochemical assay

Glycerol content was analysed directly from plasma using ELISA detection kit (10010755, Cayman Chemical Company). Samples were read at 520 to 540 nm after 15 min incubation at room temperature. Measurement of enzyme activity was done using frozen liver samples homogenised in 9 volumes of ice-cold extraction buffer (20·mmoles l⁻¹ imidazole, 5.0·mmoles l⁻¹ EGTA, 5.0·mmoles l⁻¹ EDTA, 10·mmoles l⁻¹ mercaptoethanol, 50·mmoles l⁻¹ NaF and 0.1·mmoles l⁻¹ phenyl methyl sulfonyl fluoride, pH·7.4) and centrifuged at 10000 g for 10·min at 4 °C. All enzyme activities were determined spectrophotometrically with crude homogenate at 20 °C in a UV-VIS spectrophotometer (UV-1, Thermo scientific, England). Enzyme activities determined at 340·nm were calculated, based on the millimolar extinction coefficient of 6.22. All enzymes were expressed in unit per gram. One unit of enzyme activity was defined as the amount of enzyme that catalysed the hydrolysis of 1 μ mole of substrate per minute at assay temperature. All chemicals were purchased from Sisco Research Laboratories Pvt. Ltd. (India). Assay conditions were as follows:

Glycerol-3-phosphate dehydrogenase (GPDH): Assay medium contained Imidazole (20 mM, pH 7.6, 0.15 mM NADH, the reaction was initiated by 2.0 mM dihydroxyacetone phosphate (Treberg *et al.*, 2002b).

Pyruvate kinase (PK): Assay medium contained 1 mM ADP, 7 mM MgCl₂, 50 mM KCl, 0.12 mM NADH, 10 mM EDTA and 3 U ml⁻¹ LDH in 50 mM Imidazole-HCl buffer, pH 6.5. The reaction was initiated with 1.6 mM PEP (Rosa *et al.*, 1994).

Lactate dehydrogenase (LDH): The reaction solution was 100 mM sodium potassium phosphate buffer (pH 7.0), containing 69 mM pyruvate-sodium salt and 0.1 mM NADH-sodium salt. Activities were assayed at the absorbance of 340 nm (Jones and Sidell, 1982).

Phosphoenolpyruvate carboxykinase (PEPCK): The reaction mixture contained 0.008 U of enzyme, 100 μM of Imidazole-HCl buffer (pH 6.6), 2 μM of MnCl₂, 1M of GSH, 1.25 μM of sodium IDP, 50 μM of KHCO₃, 2.5 μM of NADH, 1.5 μM of PEP and 2 units of malate dehydrogenase in a volume of 1 ml. The final pH was 6.9 - 7.1 (Ballard and Hanson, 1967).

Dihydroxyacetone phosphate (DHAP): Assay medium contained 50 mM triethanolamine buffer (pH 7.5) and 0.6 mM reduced nicotinamide-adenine dinucleotide (NADH) and 100-200 μl. Samples were read after 10 min before adding 1.0 IU ml⁻¹ of GPDH (Driedzic *et al.*, 2006).

Alanine aminotransferase (AlaAT): Assay medium contained Imidazole (50 mM; pH 7.4), 0.05 mM pyridoxal phosphate, 0.2 mM NADH, 2U ml⁻¹ lactate dehydrogenase, 200 mM alanine. The reaction was initiated by adding 10 mM α-ketoglutarate (α-KG) (Treberg *et al.*, 2002a).

Aspartate aminotransferase (AspAT): Assay medium contained Imidazole (50 mM; pH 7.4), 0.05 mM pyridoxal phosphate, 0.2 mM NADH, 1.0 U ml⁻¹ malate dehydrogenase and 30 mM aspartate. The reaction was initiated by adding 10 mM α-ketoglutarate (α-KG) (Treberg *et al.*, 2002a).

Data analysis

All values are expressed as mean±SE. Comparison among groups was performed using Student's *t*-test and differences were considered statistically significant at $p < 0.05$. All statistical analyses were done using the SPSS package Ver.19.

Results

Plasma glycerol

The mean concentration of plasma glycerol increased significantly, by approximately four fold, in *S. richardsonii*

(2.07±0.02 mmoles l⁻¹) and sixfold in *B. bendelisis* (1.54±0.02 mmoles l⁻¹) over a period of 96 h, maintained at 5 °C when compared with control condition (0.51±0.02 mmoles l⁻¹ for *S. richardsonii*; 0.23±0.01 mmoles l⁻¹ for *B. bendelisis*) at ambient temperature (15-16 °C) (Fig. 1a).

Glycolytic enzymes

Glycerol-3-phosphate dehydrogenase (GPDH) is reportedly involved with glycerol synthesis catalysing the process of dihydroxyacetone phosphate (DHAP) conversion to glycerol. The GPDH increased significantly ($p < 0.05$) in cold acclimation (5 °C) in both the species (peak values: *S. richardsonii*: 9.45±0.02 U g⁻¹; *B. bendelisis*: 6.82±0.02 U g⁻¹) over a period of 96 h as compared to the ambient/control, which was measured as 8.73±0.01 U g⁻¹ and 5.41±0.03 U g⁻¹ (Fig. 1b). Other enzymes involved in the glycolytic pathway such as phosphoenolpyruvate carboxykinase (PEPCK), lactate dehydrogenase (LDH) and pyruvate kinase (PK) have shown contrasting trends. The LDH and PEPCK are involved in the gluconeogenesis process. The PEPCK showed significantly ($p < 0.05$) increasing trend after 48 h of cold acclimation in *S. richardsonii* (peak value: 0.38±0.02 U g⁻¹) while, in case of *B. bendelisis* it is significantly higher ($p < 0.05$) after 24 h (peak value: 0.23±0.02 U g⁻¹) (Fig. 1c). There was no statistically significant change measured in LDH activity in cold acclimation in both the species (Fig. 1d). While the level of PK decreased significantly ($p < 0.05$) at 24 h of cold acclimation and onwards in case of *S. richardsonii* and at 72 h and onwards in *B. bendelisis* (Fig. 1e). The DHAP concentrations were also measured both in control and cold acclimation conditions and showed a significant increase at 24 h onwards in both the species (peak values: *S. richardsonii*: 0.59±0.02 μmoles g⁻¹; *B. bendelisis*: 0.45±0.01 μmoles g⁻¹) (Fig. 1f).

Enzymes of amino acid metabolism

The alanine aminotransferase (AlaAT) activity level increases significantly ($p < 0.05$) after 24 h of cold acclimation in both *S. richardsonii* (peak value: 1.27±0.01 U g⁻¹) and *B. bendelisis* (peak value: 0.98±0.01 U g⁻¹) when compared with ambient condition (Fig. 1g). The level of aspartate aminotransferase (AspAT) also showed an increasing trend in both the species and significant ($p < 0.05$) increases were measured at 96 h of cold acclimation in case of *S. richardsonii* (1.06±0.01 U g⁻¹) and from 24 h onwards in case of *B. bendelisis* (peak value: 0.78±0.01 U g⁻¹) (Fig. 1h).

Discussion

The results of the present investigation indicate that both the species *S. richardsonii* and *B. bendelisis* produce

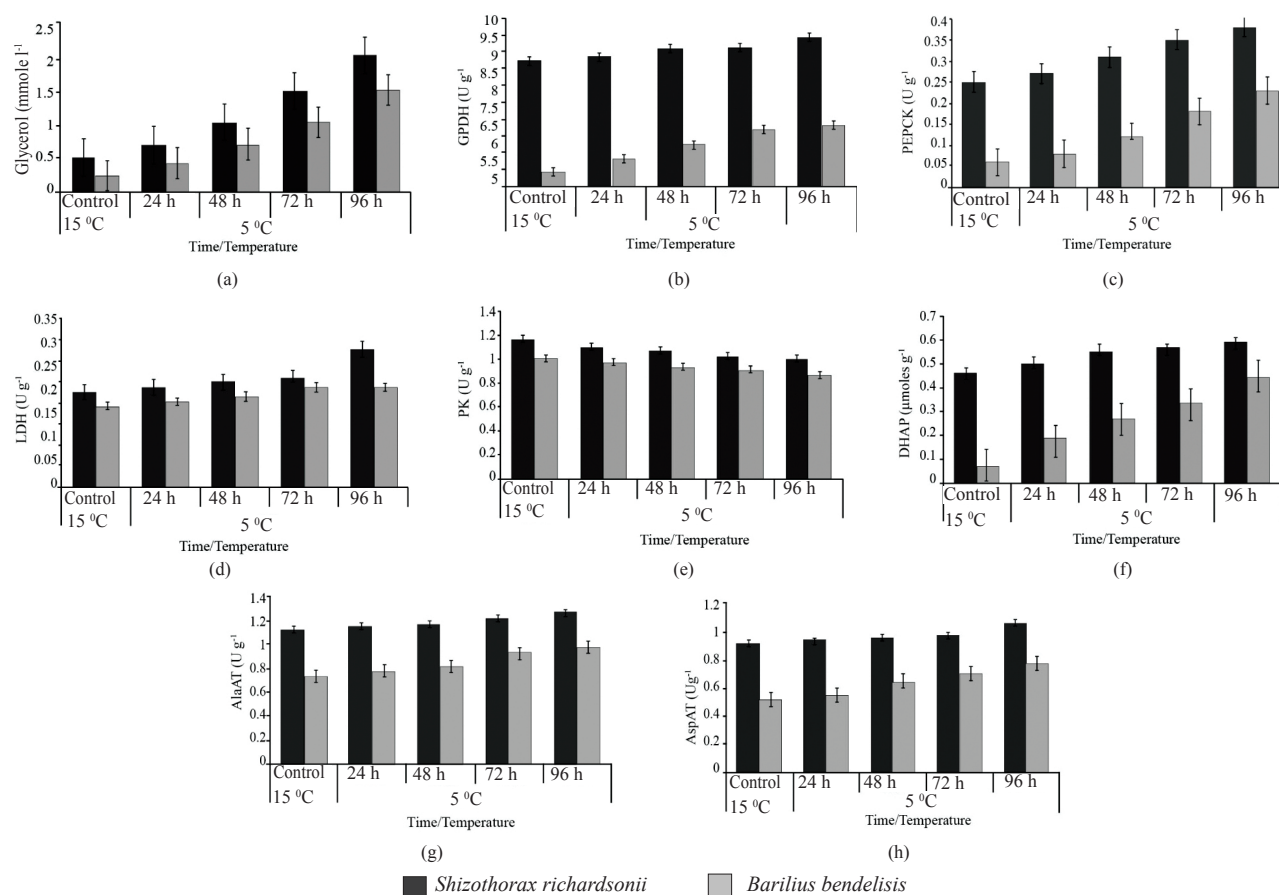


Fig. 1a. Plasma glycerol and enzyme activities measured at ambient and cold condition for *S. richardsonii* and *B. bendelisis*. (a) Plasma glycerol, (b) Glycerol-3-phosphate dehydrogenase, (c) Phosphoenolpyruvate carboxykinase (d) Lactate dehydrogenase, (e) Pyruvate kinase, (f) Dihydroxyacetone phosphate, (g) Alanine aminotransferase, (h) Aspartate aminotransferase, (*shows the significant differences from control, p<0.05)

relatively high levels of glycerol under cold condition. The specimens were caught at higher temperature (15-16 °C) and kept under their lower thermal limits (5 °C) for a period, of 4 days. During these periods, the level of blood glycerol kept increasing. The results are in conformity with earlier studies conducted with rainbow smelt, which also showed a higher level of glycerol accumulation in the blood under cold condition (Driedzic *et al.*, 1998; 2006). Furthermore, it is reported that liver is the main site for the glycerol synthesis from either glycogen or amino acid. In case of both the species, liver exhibited higher activities of glycerol-3-phosphate dehydrogenase (GPDH). The dihydroxyacetone phosphate (DHAP) which remains in equilibrium with glyceraldehyde-3-phosphate gets converted into glycerol and the process is catalysed by GPDH. The increasing concentration of DHAP coupled with higher activity of GPDH indicates that liver is the source of glycerol and carbon flow is mainly from DHAP. Similar phenomenon has been reported from earlier studies in marine rainbow smelt by Driedzic

et al. (2006). It is also reported that there are other sources of carbon for glycerol synthesis, in addition to liver glycogen (Raymond 1995; Raymond *et al.*, 1996). Amino acids are used as fuel for glycerol production *via* gluconeogenesis, referred to as glyceroneogenesis (Hanson and Reshef, 2003). The enzymes associated with amino acid degradation, alanine aminotransferase (AlaAT) and aspartate aminotransferase (AspAT) were measured at higher levels in the liver of *S. richardsonii* and *B. bendelisis* during the period of cold acclimation process than ambient condition. However, it is also important to mention here that in the present experiment the marginal increase in the level of AlaAT and AspAT was measured after a period of 72 h of cold acclimation. The use of amino acid to support higher level of glycerol production might be associated with higher level of protein diets as suggested in case of rainbow smelt which mainly feeds on animal diets (Driedzic *et al.*, 2006). Here, in case of these cyprinids, which are primarily herbivores and mainly feeds on phytoplankton and periphyton, the

required availability of amino acids for glycerol synthesis might not be sufficient for an early response, as the maintenance of glycerol level is dependent upon the intake of protein.

Phosphoenolpyruvate carboxykinase (PEPCK) is one of the key enzymes involved in the process of gluconeogenesis which can initiate the flux of carbon into the gluconeogenic pathway (Suarez and Mommsen, 1987; Treberg *et al.*, 2002b). PEPCK activity was higher in the liver of both the species and the activity of pyruvate kinase (PK) was low. This indicates that the gluconeogenesis is likely to occur *via* a typical gluconeogenic pathway *via* pyruvate carboxylase and PEPCK. The higher amino transferase activities support the view that the amino acid carbon chains enter into the gluconeogenic pathways for glycerol production. The present finding is in agreement with earlier reports in case of rainbow smelt (Treberg *et al.*, 2002b). However, in the present experiment the contrasting trends of PEPCK and PK become substantially evident after a period of 72 h of cold acclimation. There was no substantial change in the activity of lactate dehydrogenase (LDH) during the cold acclimation. LDH is involved in the supply of energy in the form of ATP for increased muscle contraction (D'Amico *et al.*, 2002). During the cold acclimation period, the fishes remain in inactive state and showed only infrequent movements. This indicates that lactate accumulation might be low during cold acclimation period.

Based on the enzyme profile studied, it is established that in freshwater cyprinids; *S. richardsonii* and *B. bendelisis* a decrease in water temperature triggers the release of glycerol in the blood plasma and also activates metabolic response for glycerol accumulation. The higher accumulation of glycerol seems to provide a kind of protection during the cold acclimation in these species. Although the experiment was restricted for 96 h of cold acclimation, none of the specimen had shown any sign of mortality and were revived in active state. However, glycerol accumulation and elevated enzyme levels in these cyprinids help in overcoming the cold stress but it is difficult to compare it with osmerid fishes, where glycerol accumulation level is much higher at -1.8 °C or freezing temperature of seawater. Ewart *et al.* (2001) have shown an increase in mRNA for GPDH in cold acclimated smelt. However, response at molecular level in these species is yet to be established.

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