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FULL LENGTH ARTICLE

Regulatory mechanism on enhancing protein synthesis in skeletal muscles of cold exposed fresh water fish (*Channa punctata*)

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Abstract *Channa punctata* varieties of fish are energetic and survive in critical environment although the molecular mechanism is not known. They were exposed to cold (4–8 °C) for 30 min, 1 h, 2 h and 4 h and the total protein contents in the liver were not significantly changed up to 4 h of cold exposure while a significantly increased protein level in the skeletal muscle was noted and maximal at 2 h. Groups of fish were exposed to Na_2HAsO_4 to examine its role on cold-induced protein synthesis in the skeletal muscle and the increased protein in the skeletal muscle was reduced significantly. The results appear to indicate that cold acclimation induces a metabolic change involving cellular protein content tissue specifically and arsenic might be involved in impairment of the cold-induced effect. To clarify the molecular mechanism, groups of fish exposed to cold for 1 h and 2 h had significantly increased RNA in the skeletal muscle compared to control fish, however, a higher level was found after 2 h of treatment and the enhanced RNA induced by cold was almost completely prevented by Na_2HAsO_4. Our findings will give a new insight into the survival process of this species while toxic arsenic prevents this cellular bioprocess.
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1. Introduction

Temperature fluctuation is a common phenomenon of the atmosphere and is involved in changes of various metabolic functions. For example, low temperature has been recognized as a major environmental sympathetic stimulus and is a stressful event that elicits different thermogenic adaptive responses in endotherms and exotherms. In mammals, including humans, the physiological responses involve changes in energy

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expenditure, heat production and dissipation, physical activity and appetite (Lowel and Spiegelman, 2000). In rodents, shivering, activation of the sympathetic axis (Spiegelman and Flier, 2001) with remarkable activity of mitochondrial uncoupling proteins (UCPs) (Boss et al., 2000; Golozoboubova et al., 2001) was reported as a pivotal mechanism. The greater the UCP concentration, the greater the capacity to uncouple mitochondrial oxidative phosphorylation so that heat is produced.

Channa punctatus is generally found in fresh water of haor, bil, river in Bangladesh. They are much energetic and survive in the critical circumstances for long time. They are the major sources of protein in the diet for human being. It is assumed that the higher energy content of this fish is caused by the increased activity of the sympathetic nerves. Peripheral tissue metabolism is affected by both environmental and chemical stimuli; however, endogenous auto regulation of metabolic processes of all species is a common biological process. Degradation of biomolecules as well as biosynthesis is the characteristics of metabolic processes. Among the peripheral tissues, the skeletal muscle and the liver play a great role in metabolic regulation. The metabolic functions in these tissues are influenced by both environmental and chemical stimuli. Liver glycogenolysis is a metabolic process yielding energy for doing mechanical work and the process is enhanced upon activation of the sympathetic nervous system. The skeletal muscle comprises both oxidative and glycolytic fibers and is therefore, metabolically important. Both adrenergic and nor-adrenergic nerves fibers are predominant in this tissue. Therefore, it is speculated that cold exposure would have effect in the regulation of metabolic functions through activation of these nerves. Although fish are exposed to various environmental stimuli, the species wants to maintain the homeostasis of the body. Adaptive thermogenesis, the dissipation of energy in the form of heat in response to external stimuli, has been implicated in the regulation of energy balance and body temperature. In shivering thermogenesis, because of the higher oxidative process, generation of ATP rather than UCP is predominant and hydrolysis of ATP yields energy useful for doing mechanical work and for living in the atmosphere. However, the molecular mechanism involving the adaptive response for this species is not clarified.

Arsenic is toxic to the living organisms. Prolonged exposure of arsenic has detrimental effects in tissues. It may impair the glycolysis as well as the oxidative processes (Tchounwou et al., 2003) and causes different types of pathogenic syndromes in rodents, fish and other organisms. Exposure of higher concentration of arsenic in water may also cause severe effects in fish and might be involved in producing cancer or other cellular effects. However, the mechanism underlying the effects of acute arsenic exposure on the regulation of oxidative and glycolytic processes in tissues of fish exposed to cold is not known. Arsenic is classified as a human carcinogen based on several epidemiological studies showing an association of arsenic exposure with cancers in lung, bladder, kidney and liver (Hughes, 2002; Tchounwou et al., 2003). Moreover, fish have long been used as sentinels for biomonitoring of aquatic environmental pollutants and are good indicators of arsenic toxicity (Tisler and Zagorc-koncan, 2002). Both cold and toxic arsenic make a critical environment where the fish survive, however, the mechanism underlying the survival process is not clarified.

2. Materials and methods

2.1. Fish

C. punctatus weighing 50–60 g were used and maintained in normal water with ambient temperature (25.0 ± 1 °C). On the day of experiment, different groups of fish were exposed to cold (4–8 °C) in the cold chamber for 30 min, 1 h, 2 h and 4 h period with full aeration and with free access to water. After cold exposure treatment, fish were quickly decapitated and the peripheral tissues including the skeletal muscle from the dorsal part and the liver were sampled carefully and weighed by a digital balance (Chyo, JL-180, China) and kept at −20 °C. Control fish were similarly used for sampling of tissues except cold exposure.

2.2. Arsenic treatment

To examine the role of arsenic on the regulation of metabolic activity involving the amount of protein and RNA in the skeletal muscle, groups of fish were exposed with arsenic compound (100 mM Na_2HAsO_4. $7H_2O$, BDH Chemical Ltd.) in cold for 1 h and 2 h. The respective other group of fish was treated with only 100 mM of Na_2HAsO_4 for 1 h in ambient temperature for determination of protein only. The tissues were sampled after the treatment similarly as mentioned above.

2.3. Assay of tissue protein content

Tissues were homogenized with pre-cooled water and were centrifuged at 8000 rpm for 10 min. The supernatants from each tissue homogenate were used as crude extract for assay of protein by using 50 μL extract. The protein content in tissue was determined by the procedure of Lowry et al. (1951). Briefly, an alkaline solution was prepared by mixing 50 mL of alkaline Na_2CO_3 solution (2% Na_2CO_3 in 0.1 N NaOH) and 1.0 mL of copper–sodium potassium tartarate solution (1 g sodium potassium tartarate and 0.5 g $CuSO_4$. $5H_2O$ were dissolved in 100 mL distilled water). Fifty microliters of tissue extract was taken in the test tube and made up to 1 mL with distilled water. For blank, 1 mL water was used in place of tissue extract. Five milliliters of alkaline solution was added to each tube and mixed well. The tubes were allowed to stand for 10 min at room temperature and 0.5 mL of diluted FCR (Commercial FCR was diluted with equal volume of water) was added and mixed well. After 30 min, the absorbance was taken at 650 nm against the blank. The protein content in each tissue was calculated from the standard graph of bovine albumin (1 mg/mL) and is expressed as g/100 g of tissue weight.

2.4. Estimation of RNA content

The RNA of skeletal muscle was estimated by the phenol–chloroform extraction method (Joseph and David, 2001). Briefly, equal volume of phenol:chloroform (10 mL:10 mL) was added to homogenized skeletal muscle in a glass tube with plastic cap and the contents mixed vigorously until an emulsion forms. The mixture was centrifuged at 5000 rpm for 5 min and the lower aqueous phase was transferred to another

tube with a pipette. The above two steps were repeated using equal volume of phenol (5 mL) and chloroform (5 mL), shaken vigorously and the volume measured. An equal volume of chloroform was added, the tube shaken and centrifuged at 5000 rpm for 5 min. The liquid phase was collected and an equal volume of 95% ethanol was mixed and kept for overnight. The precipitate was collected by centrifugation at 6000 rpm for 15 min and was washed with 15 mL of 65% ethanol. The precipitate containing pure RNA was dissolved with 2 mL distilled water and the amount of RNA was measured spectrophotometrically at 260 nm and 280 nm. The reading at 260 nm allows calculation of the concentration of nucleic acid in the sample. An OD of 1 corresponds to approximately 40 μg/mL for single stranded RNA. The ratio between the readings at 260 nm and OD of 280 nm (OD_{260}/OD_{280}) provides an estimate of the purity of the nucleic acid. Pure preparations of DNA and RNA have OD_{260}/OD_{280} values of 1.8 and 2.0 respectively.

2.5. Statistical analysis

Results of the experiments were expressed as mean and standard error of different groups. The differences between the mean values were evaluated by ANOVA followed by the paired *t*-test using SPSS software.

3. Results

3.1. Time course effect of low temperature on the regulation of protein content in skeletal muscle

To examine the role of cold exposure on the regulation of tissue protein, the fish were exposed to cold for 30 min, 1 h, 2 h and 4 h in the cold chamber. For control fish kept in ambient temperature, protein content in the skeletal muscle was 4.04 ± 0.71 g/100 g of tissue weight. After 30 min and 1 h exposure of cold, the values were 5.13 ± 0.67 g and 6.16 ± 1.19 g/100 of tissue weight respectively. Protein contents were increased significantly by 26.9% ($P < 0.05$) and 52.5% ($P < 0.05$) respectively. Fish exposed to cold for 2 h and 4 h had 9.42 ± 1.02 g and 3.28 ± 0.35 g of protein respectively in their tissues. Cold exposure stimulates the synthesis of protein significantly by 133.2% ($P < 0.01$) after 2 h while the value was reduced non significantly by 18.8% after 4 h when compared to the tissues of control fish (Fig. 1). The increased protein in response to cold acclimation might be involved in the survival process for this species of fish.

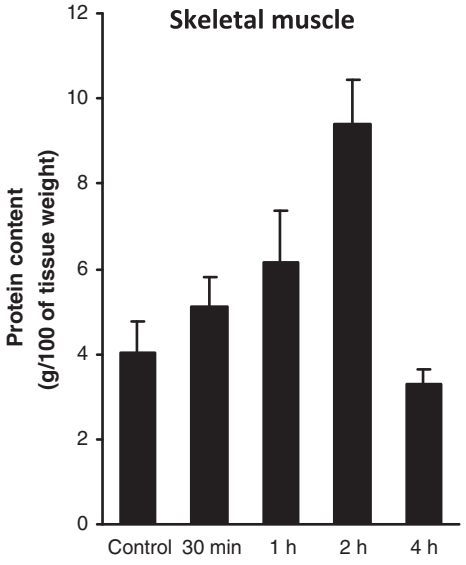

Figure 1 Effects of low temperature on protein level in the skeletal muscle of fish. The fish were exposed to cold for 30 min, 1 h and 2 h and 4 h in the cold chamber. After the treatment, the fish were immediately decapitated and sampling of tissue was performed. Control fish were similarly used except cold exposure. The data are ± SEM for 4–5 fish in each group.

3.2. Time course effect of low temperature on the regulation of protein content in liver

As shown in Fig. 2, the average protein content in the liver of fish exposed to cold for 30 min, 1 h, 2 h and 4 h was 10.87 ± 0.57 g, 10.20 ± 0.55 g, 10.15 ± 1.85 g and 10.41 ± 0.94 g respectively while for the control fish, the value was 10.63 ± 0.72 g/100 g of tissue weight. No significant changes of protein content in the liver were found up to 4 h of cold exposure and were almost similar to the control fish. The results demonstrate that cold exposure is involved in the regulation of metabolic function in the liver without alteration of tissue protein content in this species of fish.

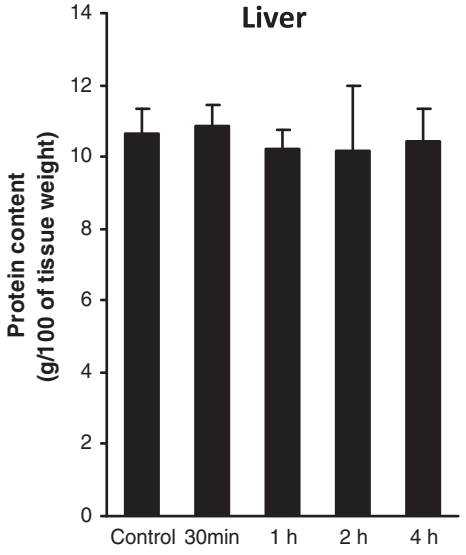

Figure 2 Effects of low temperature on protein content in the liver of fish. The fish were exposed to cold for 30 min, 1 h, 2 h and 4 h in the cold chamber. After the treatment, the fish were immediately decapitated and sampling of liver was performed. Control fish were similarly used except cold exposure. The data are means ± SE for four fish in each group. No significant changes of protein with respect to control were observed.

3.3. Role of Na_2HAsO_4 on protein content in cold-induced skeletal muscle

Groups of fish were used to examine the role of arsenic on the changes of protein in the skeletal muscle. The protein content

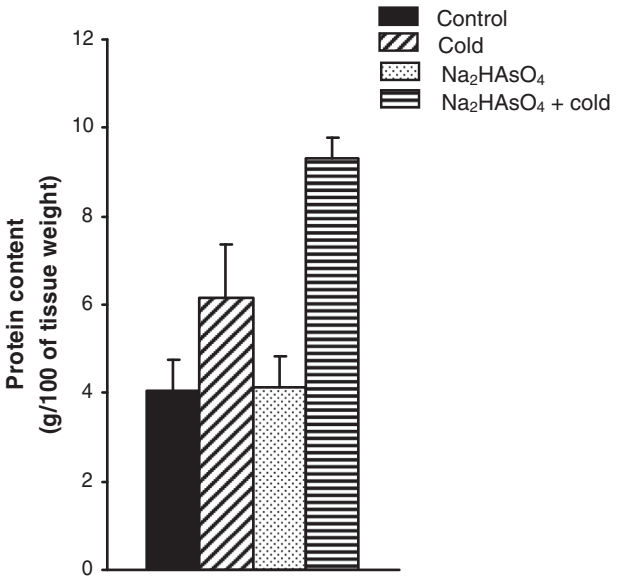

Figure 3 Effects of Na_2HAsO_4 (100 mM) on protein level in the skeletal muscle of fish. The groups of fish were treated with arsenic solution and kept for 1 h in the cold. The respective controls were treated with arsenic only while other fish were exposed to cold for 1 h. Control fish were similarly used except cold exposure. The data are means ± SE for 4–5 fish in each group.

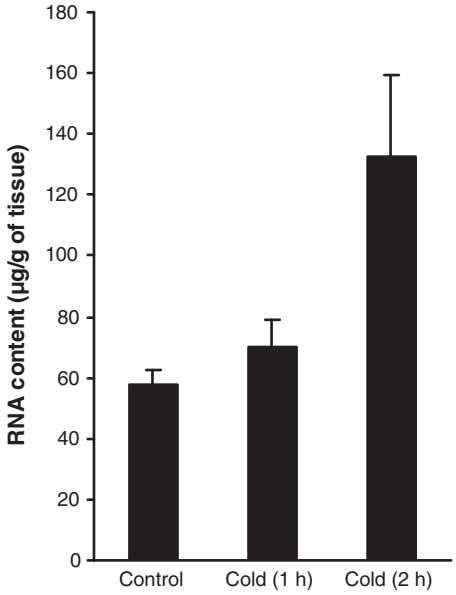

Figure 4 Effects of cold acclimation on RNA content of the skeletal muscle of fish. The fish were exposed to cold for 1 h and 2 h. After the treatment, the fish were immediately decapitated and sampling of tissue was performed. The tissues were analyzed for RNA. Control fish were similarly used except cold exposure. The data are means ± SE for three fish in each group.

of arsenic-treated fish for 1 h was 4.12 ± 0.70 g whereas for control and cold exposed fish for 1 h, the values were 4.04 ± 0.71 g and 6.16 ± 1.19 g/100 g of tissue weight respectively. The amount of protein (shown in Fig. 3) in response to cold was increased significantly ($P < 0.05$) compared to control while the value was found to be reduced (33.1%, $P < 0.1$) by Na_2HAsO_4 when compared to cold exposed fish. Groups of fish were exposed to cold with arsenic solution and the protein content in the skeletal muscle was 9.32 ± 0.47 g/100 g of tissue. The protein content in the presence of arsenic in cold was found to be increased significantly when compared to the arsenic treated- (126.2%, $P < 0.05$) and control (130.7%, $P < 0.05$) fish. The results appear to indicate that arsenic might be involved in reducing the cold induced protein synthesis in the skeletal muscle, however, both the chemical and environmental stresses seemed to cause the synthesis of stress proteins to survive in that circumstances.

3.4. Time course effect of low temperature on the regulation of RNA content in skeletal muscle

To clarify the molecular mechanism of enhancing protein in the skeletal muscle, groups of fish were exposed to cold for 1 h and 2 h and we examined the changes of RNA level in this tissue. As shown in Fig. 4, the amount of RNA in response to cold for 1 h and 2 h was 70.08 ± 9.05 and 132.70 ± 26.75 µg/g of tissue respectively while for the control, the value was 57.61 ± 5.08 µg/g of tissue. A significant 21.6% ($P < 0.05$) and 130.3% ($P < 0.01$) enhanced RNA in the skeletal muscle was found after 1 h and 2 h respectively when compared to the tissue of control fish. However, higher activity was observed after 2 h of cold. Cold exposure stimulates RNA content time dependently up to 2 h. The changes of RNA content in tissue in response to cold might be involved in the regulation of skeletal muscle metabolic functions. The alteration of RNA in this tissue is an index for characterization of the sensitivity to the environmental temperature and might be involved in the adaptive response for survival in the atmosphere for this species of fish.

3.5. Role of Na_2HAsO_4 on RNA content and RNA/protein ratio in cold-induced skeletal muscle

The amount of RNA in response to 100 mM Na_2HAsO_4 in cold for 1 h was 40.56 ± 5.24 µg/g of tissue whereas for control and the cold exposed fish, the values were 57.61 ± 5.08 and 70.08 ± 9.05 µg/g of tissue respectively (Table 1). The results demonstrated that the RNA contents had been significantly reduced (42.1%, $P < 0.05$) by Na_2HAsO_4 compared to respective cold exposed group and also to control (29.6%, $P < 0.05$). For 2 h exposure in cold, arsenic causes 41.15 ± 0.80 µg RNA and fish exposed to cold for 2 h had 132.70 ± 26.75 µg, therefore, the amount of RNA was reduced by 28.6% significantly ($P < 0.05$) by arsenic treatment when compared to control and respective cold exposed group (69.0%) ($P < 0.05$), however, higher efficiency was observed in 2 h treatment. The result shows clearly that arsenic might be a potent inhibitor on cold induced RNA synthesis in the skeletal muscle and may act through inhibition of the sympathetic nervous system.

RNA and protein concentrations as well as RNA:protein ratios (milligrams of RNA per gram protein), as traditional indicators of the *in vivo* capacity of protein synthesis, were measured in tissues of control and cold-acclimated *C. punctata*, for an evaluation of effects of long-term acclimation and evolutionary cold adaptation. The RNA:protein ratio for control

Table 1 Effects of Na_2HAsO_4 (100 mM) on RNA content of the skeletal muscle of fish. The fish were exposed to cold with arsenic solution for 1 h and 2 h. Other groups of fish were exposed to cold for 1 h and 2 h only. After the treatment, the fish were immediately decapitated and sampling of tissue was performed. The tissues were analyzed for RNA. Control fish were similarly used except cold exposure. Purity of RNA from the skeletal muscle of different groups of fish was shown.

	Control	Cold (1 h)	Cold (2 h)	Na_2HAsO_4 + cold (1 h)	Na_2HAsO_4 + cold (2 h)
RNA content (μg/g of tissue)	57.61 ± 5.08	70.08 ± 9.05[A]	132.70 ± 26.75[B]	40.56 ± 5.24[C]	41.15 ± 0.80[D]
Purity (%)	83.41 ± 3.31	92.62 ± 2.54	88.39 ± 4.10	80.38 ± 5.82	86.42 ± 0.74

The data are means ± SE for three fish in each group.
[A] $P < 0.05$.
[B] $P < 0.01$ versus control for 1 h and 2 h respectively.
[C] $P < 0.05$ versus cold (1 h).
[D] $P < 0.05$ versus cold (2 h).

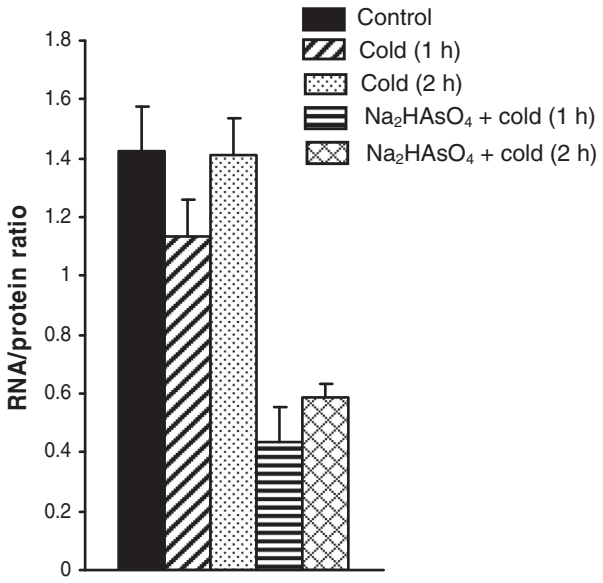

Figure 5 Effects of Na_2HAsO_4 (100 mM) on RNA:protein ratio in the skeletal muscle of fish exposed to cold for 1 h and 2 h. The control fish were used similarly except cold exposure. The data are means ± SE for three fish in each group.

was 1.425 ± 0.15 while for cold exposed fish for 1 h and 2 h, the ratios were 1.137 ± 0.12 and 1.408 ± 0.13 respectively. On the other hand, the ratios for arsenic in cold exposed tissues for 1 h and 2 h were 0.435 ± 0.12 and 0.586 ± 0.05 respectively (Fig. 5). The results demonstrated that the RNA:protein ratios had been significantly influenced by Na_2HAsO_4 as well as in cold compared to the cold exposed group.

4. Discussion

Protein synthesis is energetically expensive, accounting for 11–42% of basal metabolism in a range of ecto- and endotherms and is therefore a major component of overall animal energetics (Houlihan et al., 1995). The continual synthesis and degradation of proteins is not only vital for tissue maintenance and animal growth but is also important in allowing animals to adapt to changing environmental conditions, to replace denatured or damaged proteins, to mobilize amino acids and to allow metabolic regulation (Hawkins, 1991). In our study, fish exposed to cold had increased protein in their tissues particularly skeletal muscles. However, this might be mediated likewise by the sympathetic nervous system, since skeletal muscles have been recognized to be supplied with noradrenergic sympathetic axons that are distributed to the muscle spindles and extrafusal muscle fibers (Barker and Saito, 1981). Moreover, the skeletal muscles are thermogenic in nature. Recent investigation reveals that the thermogenesis in the skeletal muscle is caused by the higher expression of UCP-3 protein (Min et al., 2000). Cold exposure is the major sympathetic stimulus regulating metabolic functions. The thermogenesis caused by the higher expression of UCP-3 in the skeletal muscle particularly in cold environment is referred to as the nonshivering thermogenesis linked to the generation of heat directly (Duchamp and Barre, 1993). It is assumed that the increased protein in response to cold might be a survival factor for this species during environmental low temperature.

Liver is the major organ involved in metabolic regulation. The stored glycogen in the liver is influenced by the activation of the sympathetic nervous system induced by cold exposure (Thomas and George, 1975). The energy output from the liver responsible for doing mechanical work is caused by the activation of glycogenolysis process. Although UCP-2 is expressed in this tissue, however, cold exposure did not alter protein content in this tissue. It might be possible that the mechanism involving the triggering response to the synthesis of protein in the liver is different from other tissues. Kent et al. (1988) found that there was no change in either total liver DNA content or protein concentration per gram weight, following acclimation of channel catfish to a reduction in temperature. Therefore, their findings made a good illustration to support the result.

Arsenic is a potent carcinogenic toxic compound and causes impairment of several metabolic functions. The reduced protein content in the skeletal muscle in response to Na_2HAsO_4 might be due to the impairment of the sympathetic nervous system of these tissues since cold exposure stimulates the nerve activity. However, fish exposed with arsenic in cold show a significantly increased protein in these tissues, therefore, it is assumed that in these diverse adverse environments, fish want to survive by causing the synthesis of adaptive proteins and cold induced sympathetic nerve activity plays the dominant role than the arsenic exposure. The mechanism of the synthesis of protein in skeletal muscles of the variety of *C. punctatus* exposed to low temperature was clarified in the present study. In the skeletal muscle, cold acclimation significantly enhanced

RNA content time dependently and the value was maximal at 2 h, however, reduced to control level after treatment with Na_2HAsO_4. Therefore, it is generally accepted that the increased protein might be due to the higher synthesis of RNA in the cells and arsenic is involved in interaction and impairment of the synthesis. Recent findings demonstrated that inhibition of germination, plant root growth and cell division during mitosis had been noticed at higher concentrations of arsenic (Mumthas et al., 2010), therefore, the experimental results correspond to their findings. Elevated RNA in cold adapted fish may therefore be the result of enhanced RNA stability resulting from low RNA turnover rates and may not reflect enhanced energy costs. Elevated levels of mRNA and protein synthesis in turn, would support short diffusion pathways for newly synthesized protein to their final usage sites. Moreover, the increased protein synthesis could be due to the increased sympathetic nerve activity since cold exposure elicits the activation of sympathetic nervous system. Cold acclimation has been involved in inducing aerobic oxidative and glycolytic processes and since skeletal muscles are composed of both oxidative and glycolytic fibers, the increased protein may take part in the cellular metabolic process during cold acclimation. Enhanced capacities of the protein synthesis apparatus especially in the cold resemble high enzyme capacities of aerobic metabolism, which are cold compensated too, despite reduced standard and maximum metabolic rates. Such excess capacities in metabolic and protein synthesis functions may be relevant to rapid adjustment of metabolic and functional equilibria and for full metabolic flexibility in response to external and internal stimuli in the permanent cold. The extremely high capacity of the protein synthesis system in the white muscle strongly supports these conclusions.

Enabling of the protein synthesis machinery to function at very low operating temperatures *in vivo* has been suggested to be brought about by elevated tissue RNA:protein ratios (milligrams of RNA per gram protein). Accordingly, this parameter is commonly used as an indirect measure of the *in vivo* protein synthesis capacity of a tissue (Waterlow et al., 1978; Sugden and Fuller, 1991). Increased RNA:protein ratios have been found upon cold acclimation in various tissues of fish and has been interpreted to reflect cold compensation of RNA translational activities (K_{RNA} *in vivo*, defined as grams of protein synthesized *in vivo* per gram RNA per day, also known as RNA translational efficiency) (Goolish et al., 1984; Foster et al., 1993). The increase in RNA:protein ratios reflected by increased RNA levels in cold has been suggested to counteract a thermally induced reduction in RNA translational efficiency *in vivo*.

5. Conclusion

In summary, these tissues are metabolically important for energy consumption and energy expenditure. Central stimulation by cold exposure regulates peripheral metabolism probably by changing their protein concentration. The diverse metabolite regulation in response to low temperature is an index for the survival of these species and is a useful biological process. The increased protein synthesis in tissue in response to cold is correlated to the transcriptional level and arsenic plays a critical role in the impairment of this biological process.

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