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Comparative evaluation of metabolic enzymes activities in different tissues of striped catfish, *Pangasianodon hypophthalmus* (Sauvage, 1878) fingerlings reared at ambient and higher temperature

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Abstract

A 60-days feeding trial was conducted to study the effect of higher temperature (32°C) in comparison to ambient temperature (24.5°C) on different metabolic enzyme namely, aspartate aminotransferase (AST), alanine aminotransferase (ALT), adenosine triphosphatase (ATPase), acid phosphatase (ACP) & alkaline phosphatase (ALP) activities in different tissues of *Pangasianodon hypophthalmus*. AST activity significantly (p<0.05) increases in liver tissue as temperature increases, however, temperature do not significantly affect the AST activity in case of the muscle (p>0.05). The ALT activity increases significantly (p<0.05) in liver and muscle tissue as temperature increases. ATPase activity (at 37°C) in gill do not differ significantly (p>0.05) whereas ATPase activity in liver tissue decreases significantly (p<0.05) as temperature increases. ACP activity does not vary significantly at different temperature whereas, ALP activity increases significantly (p<0.05) as temperature increases in liver and intestine tissues. The present study clearly shows that there is a temperature dependent change in the activity of the different metabolic enzymes in different tissues studied in comparison to the control at ambient temperature fed with different energy level diet. The finding of present study suggests that the metabolic responses are variable at different temperature and different energy level which can affect the growth performances of this new candidate species for aquaculture.

Keywords: Aspartate aminotransferase (AST), Alanine aminotransferase (ALT), Adenosine triphosphatase (ATPase), Acid phosphatase (ACP), Alkaline phosphatase (ALP), *Pangasianodon hypophthalmus*, Fingerling

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Introduction

The *Pangasianodon hypophthalmus*, commonly known as striped catfish or Pangasius is a commercial aquaculture species. It is native to South-east Asia, where more than 90% of the production occurs in Vietnam. Because of the remarkable growth rate of this fish there has been greater enthusiasm among the fish farmers for its culture and propagation. Its production levels and distribution in global markets are now standardized to that of other top-tier established aquaculture species such as salmon, shrimp and tilapia.

Temperature is one of the most important abiotic factors which influences all biochemical reactions and therefore has a great impact on the physiology of fish. Research on the effects of temperature fluctuation and its mechanism is becoming increasingly important in the current aquaculture, especially for commercial species. Many aquaculture species are currently exposed to increasing water temperature and various aspects of thermal consequences is being studied by many researchers in different fishes inhabiting different climatic regions of the world (Pörtner and Knust, 2007; Lorentzen, 2008; Lough and Hobday, 2011; Ranjan *et al.,* 2018). Generally, fish get acclimated when kept at an altered temperature regime for a sufficiently long duration. Most enzymes that are responsible for acclamatory responses in fish are primarily associated with pathways of energy production (Schreck, 2000). Fishes are poikilothermic animal and increasing water temperatures causes an exponential increase in the metabolic rate, this combines with decreasing oxygen solubility resulting negative effect on growth performance (Blaxter, 1991; Rombough, 1997; Katersky *et al.,* 2006; Barnes *et al.,* 2011). Water temperature is the main factor influencing the rate of metabolism and energy consumption of fish. Changes in temperature affect enzyme activity, metabolic rate, growth, development and even locomotory function in the fishes (Fry, 1971). Water temperature has a direct effect on enzyme activity (Hidalgo *et al.*, 1999; Kofuji *et al.,* 2005). It has been previously demonstrated in roach (*Rutilus rutilus*) that as water temperature increases, enzymes activity and feed intake also increase (Hardewig and van Dijk, 2003). Fish require energy for all physiological processes including digestion, maintenance of cellular functions and tissue synthesis for growth and development (Cho and Watanabe, 1986; Cho and Kaushik, 1990). Energy utilisation involves complex physiological mechanisms after the ingestion of dietary nutrients (Jobling, 1994; De Silva and Anderson, 1995).

The main objective of this study was to assess the effects of high and ambient temperature on metabolic enzymes activity of *Pangasianodon hypophthalmus* fed diet with different dietary energy level.

Materials and methods

The experiment was conducted at the wet laboratory of ICAR-Central Institute of Fisheries Education, Mumbai over a period of 60 days. The laboratory work was carried out in fish nutrition, Biochemistry and Physiology laboratory of ICAR-Central Institute of Fisheries Education, Mumbai. Experimental fishes were procured from a commercial farm

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from Kolkata. The fish were acclimatized under aerated conditions for a period of 15 days at two temperatures (12 tanks at 32°C and 12 tanks at ambient temperature, 24.5°C). Acclimation of fish those who are acclimatized at 32°C was carried out by electrical heaters (fully submersible automatic aquarium heater, 300W, RS electrical heater; Zhongshan Risheng, China). The water temperature of the tank was increased at the rate of 1°C per day till the fish were acclimated at 32°C. Fish were acclimated at this temperature for further 15 days before the start of the experiments. During acclimation, fish were fed on a basal diet containing 30% crude protein. The setup consisted of 24 uniform size plastic rectangular tanks (80 cm×57 cm×42 cm, 150 L capacity) covered with perforated lids. Two hundred forty (240) fish were randomly and equally distributed and stocked into experimental tanks following 2x4 factorial design in triplicates. The juveniles of *P. hypophthalmus*, with an average weight 4.27±0.12 g; were used for the experiment. The total volume of the water in each tub was maintained at 100 L throughout the experimental period. Round the clock aeration was provided.

During the feeding trial fish were fed to apparent satiation twice daily at 9:00 am in the morning and 5:00 pm in the evening. Water temperature in the tanks was measured twice daily and ranged from 22-28°C at ambient temperature (24.35±0.21°C, mean±SE) and 32-33°C at high temperature (32.2±0.06 °C, mean±SE). Similarly, other parameters like DO, pH, free CO_2, hardness, Ammonia, Nitrite and Nitrate were also estimated periodically (APHA, 1998) to keep the water quality optimum for the sustained culture of fish.

Experimental diets

Purified ingredients such as casein (vitamin free), gelatin, dextrin, starch, cellulose, carboxymethyl cellulose (CMC), Butylated hydroxytoluene (BHT), cod liver oil, vitamin and mineral mixture, were taken for feed formulation. Four iso-nitrogenous and iso-lipidic diets with different levels of digestible Energy (Kcal $100g^{-1}$) 342.0, 379.1, 428.5 & 448.6 were prepared as shown in Table 1. The proximate composition of different experimental diets are shown in Table 2.

Table 1: Composition of purified experimental diets.

Ingredients (Kg)	T1	T2	T3	T4
Casein	350	350	350	350
Gelatin	30	30	30	30
Starch	130	200	280	300
Dextrin	20	40	80	109
Cellulose	308.8	218.8	98.8	49.8
CMC	40	40	40	40
Cod liver oil	101	101	101	101
BHT	0.2	0.2	0.2	0.2
Vitamin- mineral mix	20	20	20	20
Total	1000	1000	1000	1000

CMC=Carboxy methyl cellulose; BHT=Butylated hydroxytoluene.

Composition of vitamin- mineral mix (PREMIX PLUS) (quantity kg^{-1})

Vitamin A (55,00,000 IU); Vitamin D_3 (11,00,000 IU); Vitamin B_2 (2,000 mg); Vitamin E (750 mg); Vitamin K (1,000 mg); Vitamin B_6 (1,000 mg); Vitamin B_{12} (6 mcg); Calcium pantothenate (2,500 mg); Nicotinamide (10 g); Choline chloride (150 g); Mn (27,000 mg); I (1,000 mg); Fe (7,500 mg); Zn (5,000 mg); Cu (2,000 mg); Co (450) L-lysine(10g); DL-Methionine(10g); Selenium(125mg).

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Table 2: Proximate composition of different experimental diets

Proximate Composition	T1	T2	T3	T4
DM (g Kg^{-1})	900.98	900.19	890.51	890.44
CP (g Kg^{-1})	370.92	370.83	370.47	370.88
EE (g Kg^{-1})	90.87	90.83	90.76	90.87
Ash (g Kg^{-1})	20.64	20.75	20.92	20.68
GE (Kcal $100g^{-1}$)	466.61	467.9	467.11	466.42
DE (Kcal $100g^{-1}$)	342	379.1	428.5	448.6

DM=Dry matter; CP =Crude protein; EE =Ether extract; GE=Gross energy; DE= Digestible energy

Sample preparation

At the end of the experiment six fish were collected from each tank and anesthetized with clove oil (50 μL L^{-1}). Fish were then dissected and the tissues viz., liver, intestine, gill and muscle, were immediately removed. A 5% tissue homogenate was prepared in chilled 0.25 M sucrose solution by Teflon coated mechanical homogeniser (REMI Equipments, Mumbai, India). The whole procedure was followed in ice cold condition. Homogenized samples were centrifuged at 5000 rpm for 10 min at 4^0C. The supernatant was collected in 5ml tube and stored in a deep freezer (-20 ^{0}C) for enzyme assay. Quantification of protein of the different tissues was carried out by using the Bradford method (Bradford, 1976).

Metabolic enzymes, aspartate aminotransferase and alanine aminotransferase

Aspartate aminotransferase (AST) activity of liver and muscle tissues of *P. hypophthalmus* was assayed as described by Wooton (1964). The substrate comprised of 0.2 M DL- aspartic acid and 2 mM α-ketoglutarate in 0.05 M phosphate buffer (pH 7.4). In the treatment and control tubes, 0.5 ml of substrate was added. The reaction was started by adding 0.1ml of tissue homogenate supernatant in the treatment tube. The assay mixture was incubated at 37^0C for 60 minutes. The reaction was terminated by adding 0.5ml of 1mM 2, 4 dinitrophenyl hydrazine (DNPH). In the control tubes the enzyme source was added after addition of DNPH solution. The tubes were held at room temperature for 20 minutes with occasional shaking. Then 5 ml of 0.4 mLNaOH solution was added and the contents were thoroughly mixed. After 10 minutes, the OD was recorded at 540nm against blank. The AST activity was expressed as nanomoles oxaloacetate formed mg^{-1} $protein^{-1}$ $minute^{-1}$ at 37°C. The procedure adopted for the estimation of Alanine aminotransferase (ALT) activity was same as that for AST activity estimation except that the substrate comprised of 0.2 M D, L- alanine instead of aspartic acid. The ALT activity was expressed as nanomoles pyruvate formed mg^{-1} $protein^{-1}$ $minute^{-1}$ at 37 °C.

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Adenosine triphosphatase

Adenosine triphosphatase (ATPase) activity in gill and liver tissues was determined by modified method of Post and Sen (1967). The reaction mixture consists of 0.1 M Tris-HCl buffer (pH 7.8), 100 mM NaCl, 20 mM KCl, 3 mM $MgCl_2$, 5 mM ATP and 0.1 ml of tissue homogenate. The mixture was incubated for 15 min and the reaction was terminated by the addition of 1 ml 10% trichloroacetic acid. After centrifugation at 5000 rpm for 10 minutes 1 mL of supernatant was taken for estimation of inorganic phosphorus. The inorganic phosphorus was estimated by the method of Fiske and Subbarow (1925) which comprises of 1 ml supernatant, 1 mL of ammonium molybdate, 2.5 mL of distilled water and 0.5 mL of ANSA (8-Anilino-1-naphthalenesulfonic acid ammonium salt). The reaction mixture was incubated for 5 minutes and then OD was taken at 660 nm. The ATPase activity was expressed as nanomoles Pi released min^{-1} mg^{-1} protein at 37 ^{0}C.

Alkaline phosphatase and Acid phosphatase

Alkaline phosphatase (ALP) activity in liver and intestine tissues were determined as described by Garen and Levinthal (1960) .The assay mixture consisted of bicarbonate buffer (0.2 M, pH 9.5), 0.1 M $MgCl_2$, tissue homogenate and freshly prepared 0.1 M para-nitrophenyl phosphate as substrate. Optical density (OD) was recorded at 410 nm. The activity was expressed as nanomoles p-nitrophenol released min^{-1} mg^{-1} protein at 37°C. Acid phosphatase (ACP) activity was estimated using the same method as ALP, except that acetate buffer (0.2 M, pH 5.0) was used in place of bicarbonate buffer.

Statistical analysis

Statistical analysis of the results was done by two-way ANOVA (Snedecor and Cochran, 1967). Significant difference among means was determined by Duncan's multiple range test (Duncan, 1955) using 2×4 factorial ANOVA via SPSS 17.0. All data presented in the text, and tables are means±standard error of mean and statistical significance for all statistical tests was set at $p<0.05$.

Results

Aspartate aminotransferase (AST)

The AST activity of liver and muscle tissues are given in the Table 3. The two-way ANOVA analysis showed that AST activity in liver varies from 18.01 to 26.51 and in muscle varies from 15.49 to 27.17 nanomoles of sodium pyruvate released min^{-1} mg^{-1} protein at 37°C. Dietary energy and temperature in individual has a significant effect ($p<0.05$) on the AST activity in liver, whereas in case of muscle dietary energy activity significantly affect the AST activity but temperature do not significantly affect the AST activity of muscle ($p>0.05$).The combined effects of dietary energy and temperature is evident as it significantly ($p<0.05$) affect the AST activity in liver and muscle tissues.

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Table 3: Aspartate aminotransferase (AST), Alanine aminotransferase (ALT) and Adenosine triphosphatase (ATPase) activity in different tissue of *Pangasianodon hypophthalmus* fed with different experimental diet at different temperature.

Dietary energy (Kcal 100g^{-1})	Temperature	AST (GOT)		ALT (GPT)		ATPase	
		Liver	Muscle	Liver	Muscle	Gill	Liver
342.0	32°C	25.10^{a}	24.00^{ab}	13.07^{c}	1.31^{e}	332.03^{a}	135.72^{a}
	Ambient	18.26^{b}	23.45^{ab}	3.11^{f}	2.63^{d}	321.90^{ab}	191.99^{c}
379.1	32°C	26.51^{a}	23.57^{ab}	10.881^{d}	2.61^{d}	206.98^{d}	212.69^{a}
	Ambient	19.31^{b}	25.18^{ab}	10.65^{de}	3.76^{cd}	310.34^{bc}	206.37^{bc}
428.5	32°C	24.79^{a}	15.49^{c}	8.84^{de}	6.81^{b}	294.73^{c}	240.68^{a}
	Ambient	18.01^{b}	27.17^{a}	8.55^{e}	3.96^{c}	297.01^{c}	253.77^{ab}
448.6	32°C	19.66^{b}	21.04^{b}	21.75^{a}	10.43^{a}	306.01^{bc}	158.45^{a}
	Ambient	18.48^{b}	15.82^{c}	17.33^{b}	6.24^{b}	184.22^{e}	195.18^{ab}
SEM		0.775	1.477	0.715	0.412	5.552	7.912
ANOVA Table		p-value					
Dietary Energy		S	S	S	S	S	S
Temperature		S	NS	S	S	NS	S
Dietary Energy× Temperature		S	S	S	S	S	S

All values are Mean ± SEM, n=3. Values in the same column with different superscript letters are significantly different (p<0.05). SEM, Standard Error of Mean; S, Significant, NS, Not Significant.
AST: activity expressed as nanomoles of sodium pyruvate released min^{-1} mg^{-1} protein at 37°C; ALT: activity expressed as nanomoles of oxaloacetate released min^{-1} mg^{-1} protein at 37°C; ATPase: activity expressed as nanomoles of Pi released min^{-1} mg^{-1} protein at 37 °C.

Alanine aminotransferase (ALT)

The ALT activity of different tissue viz. liver and muscle are given in the Table 3. The two-way ANOVA analysis showed that ALT activity in liver varies from 3.11 to 21.75 and in muscle it varies from 1.31 to 10.43 nanomoles of oxaloacetate released min^{-1} mg^{-1} protein at 37 °C. The ALT activity differ significantly (p<0.05) in liver and muscle tissues at different energy level, different temperature individually as well as in combination.

Adenosine triphosphatase (ATPase)

The ATPase activity of gill and liver tissues are given in the Table 3. The two-way ANOVA analysis showed that ATPase activity in gill varies from 184.22 to 332.03 and in muscle it varies from 135.72 to 255.37 nanomoles of Pi released min^{-1} mg^{-1} protein at 37°C.The activity of ATPase in gill varies significantly at different energy level and at interaction of dietary energy level and temperature. However, at different temperature ATPase activity in gill do not differ significantly (p>0.05).The ATPase activity in liver differ significantly at all the combination i.e., at different energy level, at different temperature and in the combination of different energy level and temperature as well.

Acid phosphatase

The ACP activity of liver and intestine tissues is given in the Table 4. The two-way ANOVA analysis showed that ACP activity in liver varies from 3.75 to 8.66 and in intestine it varies from 1.49 to 9.48 nanomoles p-nitrophenol released min^{-1} mg^{-1} protein at 37 °C. The activity of ACP in liver and intestine do not vary significantly at different temperature and at interaction of dietary energy level and temperature. However, at different energy level ACP activity in liver and intestine differ significantly (p<0.05) and following a trend that as energy level in the diet of fish increases the activity of ACP also increases.

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Alkaline phosphatase

The ALP activity of liver and intestine tissues is given in the Table 4. The two-way ANOVA analysis showed that ACP activity in liver varies from 55.50 to 77.80 and in intestine it varies from 61.64 to 120.27 nanomoles p-nitrophenol released min^{-1} mg^{-1} protein at 37 °C. The activity of ALP in liver and intestine varies significantly at different temperature and at dietary energy level. However, interaction of dietary energy and temperature significantly affect the ALP activity in intestine but not in the liver.

Table 4: Acid phosphatase (ACP) and Alkaline phosphatase (ALP) activity in different tissues of *Pangasianodon hypophthalmus* fed with different experimental diets at different temperatures.

Dietary energy (Kcal $100g^{-1}$)	Temperature	ACP		ALP	
		Liver	Intestine	Liver	Intestine
342.0	32°C	4.82^{cd}	1.59^{d}	63.89^{b}	83.68^{f}
	Ambient	3.75^{d}	1.49^{d}	55.50^{c}	61.64^{h}
379.1	32°C	5.21^{bcd}	4.32^{c}	57.56^{bc}	92.48^{d}
	Ambient	6.13^{bc}	3.74^{c}	59.44^{bc}	64.61^{g}
428.5	32°C	6.00^{bc}	7.36^{b}	61.08^{bc}	109.04^{c}
	Ambient	6.62^{b}	7.79^{b}	56.36^{bc}	86.14^{e}
448.6	32°C	8.66^{a}	9.48^{a}	77.80^{a}	120.27^{a}
	Ambient	8.47^{a}	8.76^{a}	74.78^{a}	112.90^{b}
SEM		0.515	0.287	2.354	0.744
ANOVA Table		p-value			
Dietary Energy		S	S	S	S
Temperature		NS	NS	S	S
Dietary Energy×Temperature		NS	NS	NS	S

All values are Mean±SEM, n=3. Values in the same column with different superscript letters are significantly different (p<0.05).SEM, Standard Error of Mean; S, Significant; NS, Not Significant.
ACP: activity expressed as nanomoles p-nitrophenol released min^{-1} mg^{-1} protein at 37°C,
ALP: activity expressed as nanomoles p-nitrophenol released min^{-1} mg^{-1} protein at 37°C.

Discussion

Temperature is a major determinant of physiology of fishes, as it affects physiological processes involved in many biochemical reactions occurring in the fish. In the present study, the activities of transaminase enzymes both AST (Liver) and ALT (liver and muscle) increased with increasing acclimation temperatures. This suggests that high temperature creates higher free amino acid mobilization (Das, 2002), which in turn might have produced glucose to cope up with the stress, via the process of higher gluconeogenesis (Das *et al.*, 2006). Similar observations have been reported in *L. rohita* (Das *et al.*, 2006) and *C. carpio* (Verma *et al.*, 2007; Ahmad *et al.*, 2011). The rise in the AST activity in the liver and muscle tissues at higher temperature may be a consequence of increased utilization of the product of citric acid cycle for production of energy (ATP) required for other physiological activities. In agreement with this observation, significant increase in the ATPase activity in the liver, of *P. hypophthalmus* acclimated at higher temperatures indicate increased ATP production for satisfying the energy at higher temperature.

ATPase is a membrane-bound enzyme, which maintains ions equilibrium across cell membranes, and the disruption of this equilibrium often results in physiological dysfunction (Kong *et al.*, 2007). ATPase is responsible for the transport of ions

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through the membrane and regulate Na+/K+ gradient along the cell membrane and various intracellular functions and help instant release of energy (Richards *et al.*, 2003; Chatterjee *et al.*, 2010). The main function of transmembrane ATPase is to provide energy for ions transportation across membranes and thus maintain ion homeostasis in the cytoplasm. ATPase activity is sensitive to changes in environmental variables like temperature (Morrison *et al.*, 2006) to meet the increased energy demand by fish. The increase in the ATPase activity with increasing acclimation temperatures indicated that there was no depletion of ATP as temperature increased which demonstrated the role of ATPase in the regulation of energy balance in fish (Love, 1980). Verma *et al.* (2007) also observed increased ATPase activities in *C. carpio* after thermal acclimation. Previous studies suggested that ATPase activity is weakened in fishes at low temperatures and new ATPase are synthesized to compensate for this weakened activity (Hochachka and Somero, 1984; Kong *et al.*, 2005). The increased activity of ATPase at higher temperature is believed to be the result of ATPase compensation for low temperatures.

Acid phosphatase (ACP) is a ubiquitous enzyme that hydrolyses the phosphomonoesters, and catalyze the liberation of inorganic phosphate from organic phosphate esters and help in maintaining buffer system in blood in acidic condition (Suter *et al*., 2001). There was no significant change observed in the ACP activity in liver and intestine tissue as temperature increases suggesting that the normal function of the tissues as fish are not under oxidative stress and there is no tissue damage happened due to increased temperature (Biber *et al*., 1981).

Alkaline phosphatase (ALP) is a zinc-containing metallo-enzyme which plays a key role in phosphorus metabolism. The increased ALP activity in liver and intestine tissues, with increasing acclimation temperatures observed in the present study could be attributed to the hydrolysis of high-energy phosphate bonds to liberate phosphate ions to combat high metabolic rate at high temperature (Verma *et al.*, 2007). Similar results were reported in Mrigal, *Cirrhinus mrigala*, when subjected to increasing acclimation temperatures (Das, 2004).

The biochemical analysis of a set of enzymes, which play vital roles in cellular function, not only explains the behavioural observation, but also acts as a functional biomarkers to monitor the acclimation of fishes. Increased temperature increases the energy demand as well as metabolism. The present study clearly shows that there is temperature dependent change in activity of different metabolic enzyme in different tissues studied. The finding suggests that the metabolic responses are variable and accordingly, physiological and metabolic adjustments have a critical limit of adjustable temperature range of this new candidate species for aquaculture.

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