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Research Article

STUDIES ON BIOCHEMICAL RESPONSES OF TABLE SIZED *LABEO ROHITA* (HAMILTON, 1822) TO THE THERMAL EXPOSURE AT CRITICAL MAXIMUM TEMPERATURE (CT_{MAX})

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ABSTRACT: Water temperature of aquatic bodies is gradually getting warmer and may pose threat for survival, health and production of fish in future. The critical temperature maximum (CT_{max}) is the tool to estimate the upper limit of thermal tolerance of fish but information on biochemical events occurring at CT_{max} are limited for our selected fish, *Labeo rohita*. In the present study, biochemical alteration of table sized *Labeo rohita* (Rahu) was examined at CT_{max} (42.67±0.53ºC) against fish kept at acclimation temperature (30.5±1.0ºC) by increasing water temperature continuously at the rate of 0.28°C minute^{-1}. Significant alteration was observed for all studied biomolecules with increased glucose and triglycerides and declined protein and cholesterol in serum at CT_{max}. Hormones also altered with increased value for thyroid hormones and decreased value for cortisol, however the alteration was significant only for T4. In liver glucose, triglycerides and cholesterol level was higher but protein was less at CT_{max}. Increased transaminase enzymes activities (GPT and GOT) in liver may have increased the production of the glucose from tissue protein. At CT_{max}, alteration in metabolic activities (higher amount of glucose and triglycerides in serum) for energy production and onset of heat shock responses (higher expression of hsp70 gene in liver) were occurred in Rahu. In this study, CT_{max} was 42.67±0.53ºC and warming tolerance was 12ºC indicating that Rahu can tolerate sudden increment of water temperature of a few degrees beyond present habitat temperature, although for few moments.

Key words: *Labeo rohita*, CT_{max}, Hsp70, Glucose, Thyroids hormones,

INTRODUCTION

Fisheries and aquaculture sectors are providing quality protein, livelihood and other benefits to billions of humans globally. Fish are ectothermic aquatic animal and their survival and physiological performance depend on habitat temperature (Ficke *et al*. 2007). Habitat temperature increment due to global warming (Ficke *et al*. 2007) and regional erratic intense summer may adversely affect fish (Wedemeyer *et al*. 1999) which may potentially be reflected in production from fisheries and aquaculture industry leading to various protein-energy related malnutrition to human in coming future (Brown and Funk 2008).

The upper temperature tolerance limit (UTTL) of fish species is the maximum temperature in which the species were registered to live (Daniel *et al*. 2008). Various tools are used to study the UTTL (Beitinger and Beninett 2000) but critical temperature maximum (CT_{max}), a dynamic method, is one of the most preferred tools (Lutterschmidt and Hutchison 1997). CT_{max} is maximum the temperature where fish lose it balance when temperature is increased at constant rate from habitat temperature (Beitinger and Beninett 2000). Climate scientists have proposed that the temperature of Earth will be increased gradually (maximally 4.8 °C for 100 years) in coming years along with some abrupt fluctuation in temperature (IPCC 2014).

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Temperature exposure induces different physiological and biochemical changes in fish that may be reflected in altered metabolism and other physiological processes. Different biomolecules such as glucose, protein, triglycerides, cholesterol, calcium and hormones such as cortisol and thyroids in blood are important on these aspects (Aggarwal and Upadhyay 2012, Majhi and Das 2013). Liver is a vital metabolic organ and expected to play various important roles under thermal stress (Bechmann *et al*. 2012).

Labeo rohita (Hamilton, 1822), locally known as rahu, is one of the major cultured species in Indian subcontinent, mostly preferred among Indian major carps (IMC) and nutritionally enriched (Babu *et al*. 2013). Thermal tolerance of *L. rohita*, were already assessed by researchers (Das *et al*. 2005), however information on the aspects of metabolic, hormonal and stress protein, heat shock protein 70 (hsp70) were limited at CT_{max}. Different outcomes of biochemical impacts of thermal exposure may be expected when heat is provided at faster rate, necessitating the studies on biochemical responses of selected species at UTTL. On this background, the present study was conducted with the objective to understand the biochemical responses of table sized *Labeo rohita* at CT_{max} temperature.

MATERIALS AND METHODS

Acclimation of fish in laboratory condition

The experiment was conducted during April-May, 2014. Live healthy rahu samples of similar size (weight: 100±15 g; length: 21±2 cm) were collected from local fish pond (22°46'31.8"N, 88°20'29.6"E) and acclimated in cemented water tank (2.5 ft x 5 ft x 2.5 ft) with flow through system in ICAR-Central Inland Fisheries Research Institute, Barrackpore, Kolkata, India for 30 days at 30.5±1.0ºC. Fish were fed with tubifex at the rate of 6% of body weight twice daily and the tank was cleaned on every alternate day. Water quality was monitored every week of the acclimatization period using Multiprobe meter (Hach, HQ40D), portable turbidity meter (2100Q) and Pocket Colorimeter™ II-Orthophosphate (5870006). Water quality during acclimation was temperature: 29.0 - 31.0 ºC; pH: 7.15 - 7.49; dissolved oxygen: 5.35 - 5.70 mg L^{-1}; specific conductivity: 362-377 µS cm^{-1}; oxidation reduction potential: 161-175 mV; turbidity: 2.70-3.00 NTU; alkalinity: 80-150 mg L^{-1}; hardness: 270-285 mg L^{-1}; nitrate: 0.182-0.193 mg L^{-1} and phosphate: 0.50-0.60 mg L^{-1}. The water quality was suitable for healthy maintenance of fish.

Assessment of CT_{max} and collection of fish sample

Four fish were transferred to temperature-controlled aquarium (Suan, Kolkata, India) set at ambient temperature, 24 hours before the start of the experiment (Barcellos *et al*. 2011). No feed was provided before 12 hours of start of the experiment. CT_{max} were determined by increasing the temperature of aquarium constantly at the rate of 0.28ºC $minutes^{-1}$ up to the critical point where fish lost their balance (Lutterschmidt and Hutchison 1997). The ultimate temperature for individual fish was noted. The experiment was conducted for four times and CT_{max} was expressed as arithmetic mean±standard deviation (n=16). Twelve fish samples each were collected randomly from both tanks, having at acclimated temperature (control) and CT_{max} (treated) temperature, for further biochemical analysis in blood and tissue.

Blood and tissue sample collection

Fish were anesthetized immediately using MS-222 (Sigma Aldrich, USA) at dosage of 150 ppm (laboratory standardized). Blood samples were collected from caudal vein and serum was prepared following WHO (2002) protocol. The liver samples were collected separately in micro centrifuge tubes (Tarson, India) for biochemical analysis and in RNAlater® solution (Ambion, Life technologies) for analysis of hsp70 gene expression. All the samples are stored at - 40ºC for further analysis.

Biochemical analysis

Analysis of biomolecules and enzymes

Biomolecules and hormones in serum were analysed using glucose, triglycerides, protein and cholesterol kit and ELISA kit of T4, T3 (Erba Mannheim Transasia Bio-medical Ltd., Germany) and cortisol (Cayman Chemical, USA) following manufacturer's protocols. Liver sample was homogenized in 0.25 M sucrose solution using motorized homogenizer (Remi, India) and centrifuged (Dynamica Velocity 18R, United Kingdom) at 6000 g at 4°C for 10 minutes. Supernatant was used for the estimation of biomolecules following manufacture's protocols.

For enzyme activity, liver samples were homogenized using 0.1 M phosphate buffer (15% w v^{-1}), pH 7, incubated for 1 hr at 4°C, centrifuged at 2500 g for 5 minutes, and supernatant used for further assay. Glutamate-pyruvate transaminase (GPT) and glutamate-oxalate transaminase (GOT) assay were done as per kit protocol (Erba Mannheim Transasia Bio-medical Ltd, India).

Table 1. Primer sequence, annealing temperature, product size and accession number.

Primer	Sequences (5' 3')	Annealing Temperatures (°C)	Product size (bp)	Accession No. (NCBI)
hsp70 F	AAC ACC CAG CTA TGT TGC CT	57	202	KF435075 (self)
hsp70 R	TGA ACTT TTG GTT TTC CTC CAT CA			
β actin F	GAC TTC GAG CAG GAG ATG G	60	138	Mishra *et al*. (2009)
β actin R	CAA GAA GGA TGG CTG GAA CA			

Real-time PCR of hsp70 gene

Total RNA was purified from liver samples using Tri Reagent (Sigma Aldrich, United States) method, purity was checked spectrophotometrically ($A_{260/280}$ and $A_{260/230}$) and quantified. After DNase treatment (Fermentas, Thermo Fisher Scientific. USA), cDNA was prepared using reverse transcriptase enzyme (RevertAid First Strand cDNA Synthesis Kit, Thermo Scientific, USA). The hsp70 gene expression was quantified by using self-designed primer and Master Mixes (Maxima SYBR Green qPCR Master Mixes, Thermo Scientific, USA) with the help of the real time PCR machine (RC480, Roche Molecular Systems, Inc., United States). The amplification protocol was: 96ºC for 2 minutes, 45 cycles of 96ºC for 20 seconds, 57.1ºC for 20 seconds, 72ºC for 45 seconds (quantification) followed by final extension at 72ºC for 3 minutes and melting curve analysis. The quantitative PCR condition for reference gene beta actin was done following the same thermal cycle except annealing temperature was 55.6°C. C_t values were transported to MS-Excel 2016 for further processing and represented as normalized comparative expression ($2^{-\Delta\Delta Ct}$) (Livak and Schmittgen 2001). The details of the primer sets were given in Table 1.

Statistical analysis

There was no significant difference among the data due to different tanks and time effects ($p>0.05$). Assuming all the data as normal and homogeneous, mean values were compared using unpaired t-test of equal variance. All the results were expressed in histogram as means ± SE. For all data, differences were considered statistically significant at condition $p<0.05$. All statistical calculation was done through GraphPad Prism 7 and MS-Excel 2016.

RESULTS AND DISCUSSION

Critical temperature maximum, CT_{max}

The upper thermal tolerance limit, CT_{max} for rahu was 42.67±0.53ºC and the acclimation temperature was 30.5±1.0 ºC. The warming temperature was approximately 12 ºC.

CT_{max} is the most preferred methodology to study upper thermal tolerance because it is quite simple, require a smaller number of samples, time efficient (Lutterschmidt and Hutchison 1997) and it resembles to natural condition (Bennett and Judd 1992). Fish lost its balance at CT_{max} temperature because the performance of the organism was approaching to minimum threshold at such higher temperature. Thermal buffer capacity of fish was measured by warming tolerance (Deutsch *et al*. 2008) which is defined as difference between the CT_{max} and the mean of current habitat temperatures (Deutsch *et al*. 2008). The maximum habitat temperature documented by us at nearby waterbodies in West Bengal during summer was 36.7°C that was below the determined CT_{max} of rahu. The determined warming tolerance of rahu in this study is about 12°C for laboratory condition and about 6°C for waterbodies in West Bengal. Rahu fish can

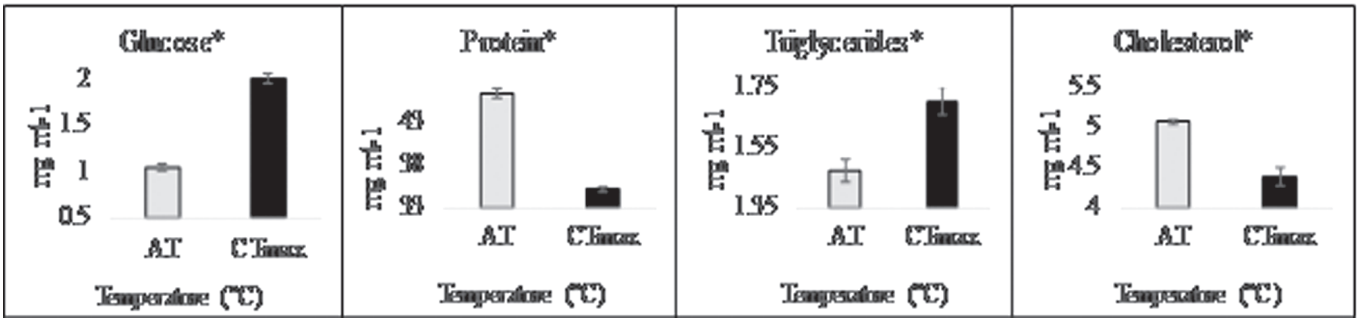

Fig. 1. Serum biomolecules.

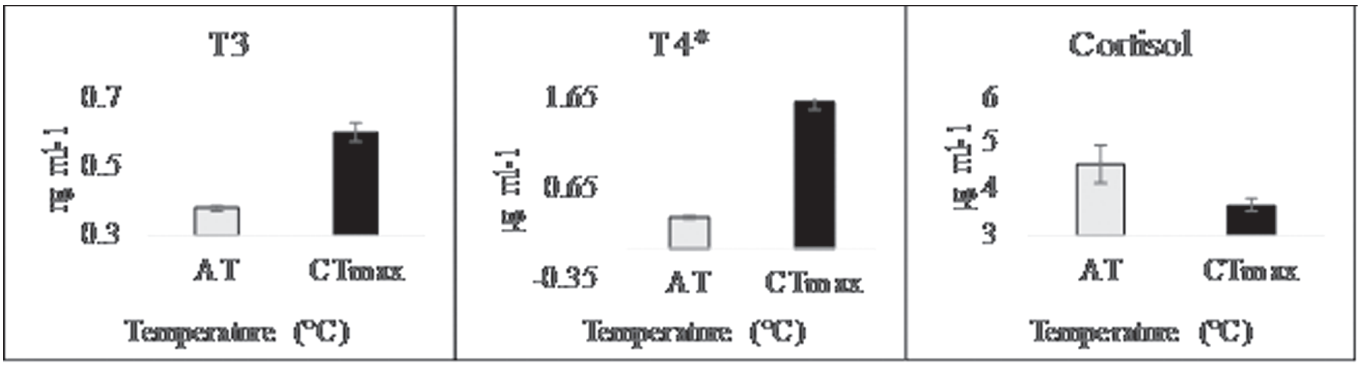

Fig. 2. Serum hormones.

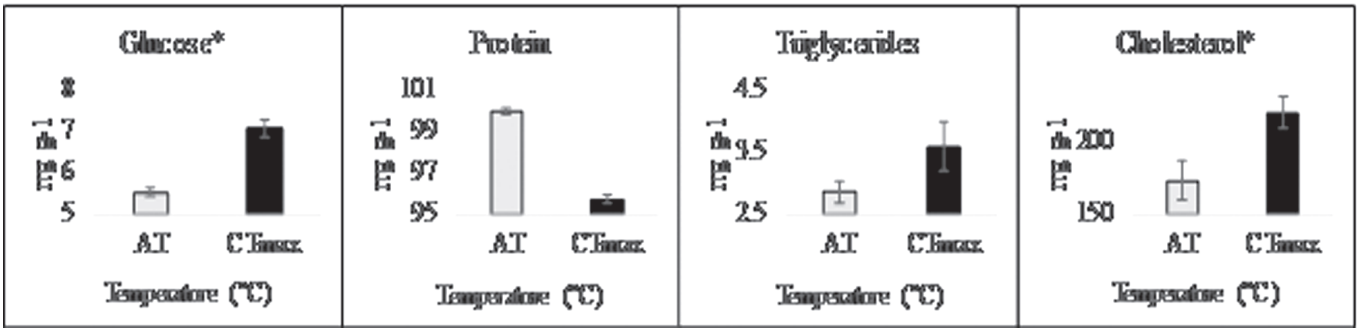

Fig. 3. Liver biomolecules.

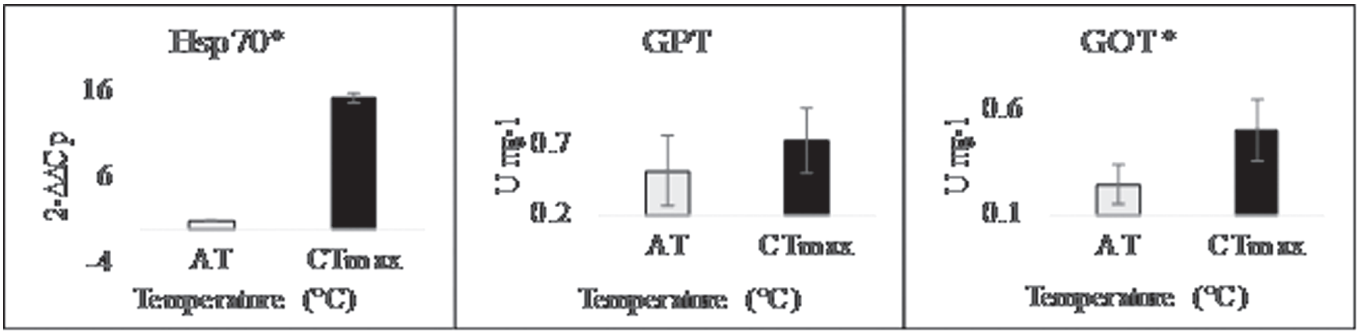

Fig. 4. Heat shock response and transaminases activity in liver.

[Legends for Fig 1 to Fig 4: AT- acclimated temperature; CTmax- critical temperature maximum; U- conversion of 1 µmol of substrate per minute; *- significant difference ($p<0.05$) in unpaired t-test of equal variance].

survive in present water bodies if there is a sudden increment in temperature of a few degrees from the present one for a few minutes, however this may not be the same for the chronic exposure at such high temperature for longer duration. The CT_{max} of rahu in our experiment differed from previous works (Das *et al.* 2004 and 2005) which may be due to the difference in rates of temperature increment (Camilo and Maria 2006), size of fish (Recsetar *et al.* 2012), temperature history (Das *et al.* 2004) and geographical location (Sorte *et al.* 2011).

Biochemical response

Significant ($p<0.05$) alteration was observed for all studied biomolecules in serum at CT_{max}. Both glucose and triglycerides increased whereas protein and cholesterol declined in serum at CT_{max} (Fig. 1). Hormones also altered at CT_{max} with increased value for thyroid hormones (T4 and T3) and decreased value for cortisol, however the alteration was significant ($p<0.05$) only for T4 (Fig. 2). Glucose ($p<0.05$), triglycerides ($p> 0.05$) and cholesterol ($p<0.05$) level was higher and protein ($p>0.05$) was less in liver at CT_{max} (Fig. 3). Gene expression of hsp70 was significantly ($p<0.05$) higher at CT_{max}. Enzyme activities of GPT and GOT were also higher in CT_{max} but significant ($p<0.05$) only for GOT (Fig. 4).

The overall results of biomolecules in serum revealed that the metabolism shifted towards energy production

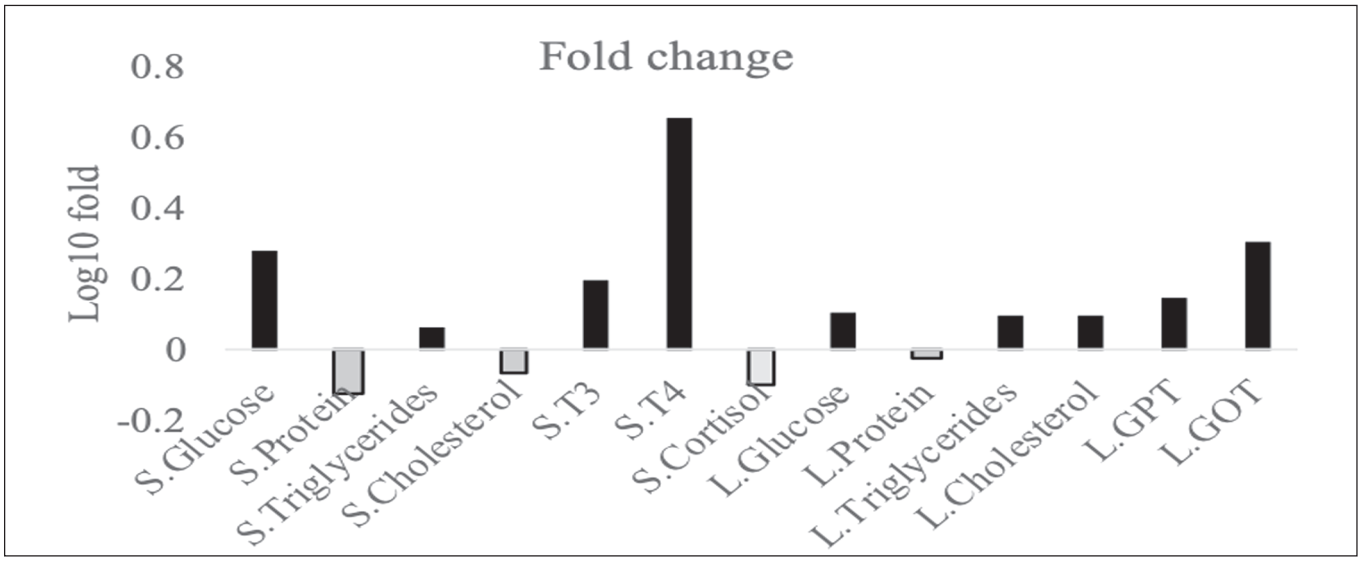

Fig. 5. Comparison of fold changes of studied biomolecules.

and higher amount of glucose in serum indicated rahu was under thermal stress (Porchas *et al*. 2009). The rate of temperature increment was fast and created quicker stress in fish that could be supported by response in form of more glucose molecules, as an immediate source of energy in serum (Mergenthaler *et al*. 2013). Higher level of glucose in serum and liver at CT_{max} was probably due to either gluconeogenesis or glycogenolysis as the fish were collected after 12 hours of fasting. In our study, declined protein level in liver and increased glucose and transaminase activity were observed in liver at CT_{max}. During stress, protein acted as precursor for gluconeogenesis and mobilized from tissue to provide energy (Vijayan *et al.* 1991*).* Higher level of glucose under thermal stress was supported with previous work of Das *et al*. (2002). Previously, blood glucose concentration of *Danio dangila* and *Brachydanio rerio* at CT_{max} temperature were examined by Majhi and Das (2013). In the present study, the cortisol concentration reduced, but difference was statistically non-significant and differed from previous research by Das *et al.* (2002) which may be due to the different nature of heat stress in both the studies (chronic vs. acute). Triglycerides and protein are also alternative source of energy but not as immediate as like glucose. Occurrence of higher triglyceride levels in serum as observed in our study is also supported by previous data (McDonald and Milligan 1992). Thyroid hormones played important role in stimulating mobilization of fat in adipose tissue (Pucci *et al*. 2000), therefore related with higher triglyceride levels in serum. Cholesterol is the precursor for synthesis of various hormones including cortisol (Miller 2008). Increased level of thyroid hormones (Aggarwal and Upadhyay 2012) and decreased level of cholesterol (Das *et al*. 2002) in serum observed in our study differed from previous findings which may be due to the nature of thermal stress applied.

Heat shock proteins (HSPs), the molecular chaperon, trigger the heat shock response under thermal stress for reversing the impacts of stress (Wu 1995). Higher water temperature causes protein denaturation due to breaking of various weak interactions where HSPs play the vital role to refold it (Wu 1995) to its native structure. Increase in hsp70 level due to heat stress was supported by findings of Purohit *et al*. (2014).

Fold changes of biomolecules were calculated as a ratio of values in CT_{max} and in acclimation temperature. Log_{10} of fold were plotted in histogram for better comparison of up-regulated and down-regulated responses (Fig. 5). Data revealed that only serum protein, cholesterol, liver protein, and cortisol were down-regulated whereas all other molecules were upregulated. T4, GOT, serum glucose, T3 and GPT have shown significant increment of 4.5, 2, 1.9, 1.6 and 1.4-fold respectively. Therefore, it can be stated that gluconeogenesis was increased in liver by converting protein to glucose by transaminase enzymes, GPT and GOT at CT_{max}. Higher amount of thyroid hormones in serum also signified its important metabolic role to increase blood triglycerides.

CONCLUSION

Rahu showed CT_{max} of 42.67±0.53ºC and warming tolerance of nearly 12ºC against the acclimation temperature of 30.5±1.0ºC. Therefore, rahu can tolerate sudden increment of water temperature to a certain limit in the present habitat condition only transiently. At CT_{max} rahu fight against thermal stress by increasing production of the immediate energy molecule, glucose, through gluconeogenesis from tissue protein by transaminases in liver and onset of heat shock response in the form of higher expression of hsp 70. Thyroids hormones played important metabolic roles by stimulating fat mobilization from storage tissue to increase blood triglycerides.

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