

Combined Effect of Heat Shock and Chlorine Fails to Elicit Acquired Thermal Tolerance in *Labeo rohita* Spawns

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Abstract An experiment was conducted to delineate the effect of heat shock and chlorine on the biochemical responses of *Labeo rohita* spawns for evaluating acquired thermal tolerance. Spawns were equally distributed in three different groups i.e., group 1 heat shock (37 °C) without chlorine, group 2 heat shock (37 °C) in chlorinated water and group 3 control in ambient water (28 °C). Following 1-h stress, the animal was transferred to ambient water conditions to study the recovery from stress at different sampling hours i.e., 0, 2, 4, 8, 14, 24 and 48 h. Stress parameters viz., glucose, lactate dehydrogenase, malate dehydrogenase and adenosine triphosphatase were measured for both the experimental groups and compared with the control. At the end of 48 h, the recovering spawns in the respective experimental groups were subjected to temperature higher than 37 °C in order to check for thermal

tolerance and induction of cross protection. Their survivability rates were checked after exposure to 38, 39, 40, 41 and 42 °C. The result indicated that the spawns in group 1 recovered within 24 h wherein heat shock alone imparted higher thermal tolerance in them leading to 100 % survival at 40 °C; however the fishes in group 2 did not recover even after 48 h and demonstrated only 30 % survival at 40 °C. In both experimental groups, none of the fish survived the exposure to 42 °C. The results indicate that the combined effect of heat shock and chlorine has more pronounced effect on *L. rohita* spawns and fails to develop acquired thermal tolerance.

Keywords *Labeo rohita* · Spawns · Heat shock · Chlorine · Biochemical responses · Thermal tolerance

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Introduction

Water quality particularly the temperature is one of the most important factors that affect fish physiology and survival. Every fish has an optimal range of temperature that it can tolerate, beyond which it creates thermal stress [1]. Thermal effluents discharged through cooling power plants not only have an elevated temperature but also contain chemical stress factors in the form of biocides (e.g., chlorine) used for biofouling control [2], which may affect the metabolic status of the fishes. Considerable concentrations of organochlorine residues were measured in fishes caught from riverine ecosystems [3]. The escalating trends in global temperature are compelling fishery researchers to make continuous effort to define thermal tolerance of fishes and their consequences on fish health [1]. Hence these potential polluting sources provide enough threats for the aquaculture industry thereby highlighting the importance to

delineate the combined effect of temperature and chlorine on fishes. Over the years, attention has been focused on the thermal tolerance of embryos and larvae [4] as they are more sensitive to temperature changes than adult fish [5]. Hence making it imperative, to study the potential effects of a dual stressor like temperature and chlorine on the recovery status of *Labeo rohita* spawns, one of the preferred Indian major carps.

Fishes may incur additional energetic costs associated with stress responses from physical and chemical fluctuations in aquatic systems [6]. These additional energy costs are met by alteration in the metabolism of fishes. Hence the authors analyzed different metabolic enzymes such as lactate dehydrogenase (LDH), malate dehydrogenase (MDH) and adenosine triphosphatase (ATPase). Under stressed condition, anaerobic pathway is activated to meet the energy requirement and therefore high level of lactate is being produced. Similarly, MDH activity is reported to increase under the influence of stress [7]. ATPase, a membrane bound enzyme responsible for the transport of ions through the membrane and regulation of Na^+/K^+ gradient along the cell membrane, is considered to be affected under stress [8, 9]. Hence the study of the enzymatic profile will reflect the recovery of *L. rohita* spawns from the effects of dual stressor like temperature and chlorine, thereby delineating the extent of damage occurred in the process of stress.

Thermal tolerance studies are conducted to obtain the tolerance zone of the species and to see the effect of other stressors on thermal tolerance capacity [10, 11]. Therefore there exists a need to know whether a dual stressor can cause any pronounced effect on the thermal tolerance zone of the spawns. The present study investigates the recovery pattern observed in *L. rohita* spawns when exposed to temperature induced stress in chlorinated and non chlorinated water and also the survivability rates after exposure and recovery from subsequent stress. In the present study recovery responses to temperature and chlorine have been correlated with metabolic enzymes to derive a more realistic picture on the physiological states of *L. rohita* spawns.

Material and Methods

Experimental Animals

Labeo rohita spawns (1 day old post hatching; mean \pm SE 0.0027 ± 0.001 g) procured from Khopoli fish seed farm, Government of Maharashtra were brought to the wet laboratory, Central Institute of Fisheries Education, Mumbai and were acclimated to laboratory conditions. The spawns were not fed during the experiment. Before commencing the experiment, the critical thermal maxima (CT_{max}) of *L. rohita* spawns was found to be 38 °C.

Experimental Design

A total of 4,950 spawns were stocked in three different experimental groups in triplicates (550 spawns/tank). The first group was subjected to acute heat shock at 37 °C for 1 h. The temperature was maintained using a thermostat aquarium (Suan Scientific Instruments and Equipments, Kolkata, India, 52 L water capacity, sensitivity ± 0.2 °C). These spawns were then transferred to tanks containing ambient waters (28 °C) at the end of 1 h stress. The second group was subjected to acute heat shock (37 °C) for 1 h in chlorinated water (0.08 ppm) in thermostat aquaria (52 L water capacity). These spawns were then transferred to ambient waters after 1 h stress. The third group was maintained as control, kept in ambient water conditions and was not subjected to any stress. Since no mortality was observed when the spawns were exposed to heat shock and chlorine stress, the entire spawns were subjected to recovery study. The recovery study was carried out over a span of 48 h. Round the clock aeration was provided in all the experimental containers to maintain optimum dissolved oxygen level.

Chlorine Dosage and Analysis

The chlorine level in the second group was maintained at 0.08 ppm by the addition of a sodium hypochlorite solution, HPLC grade. Chlorine level was continuously monitored by using Spectroquant chlorine test kit (1. 00 599. 0001, E Merck, Germany) with accuracy (0.001 mg/L).

Sample Preparation

Spawns from the respective replicates of each of the three experimental groups were sampled at the end of 1-h stress. Sampling was done at different time intervals such as 0 (immediately after 1 h stress exposure), 2, 4, 8, 14, 24 and 48 h. The spawns from the three replicates for the particular experiment were pooled and subjected to homogenization and was centrifuged (5,000 rpm at 4 °C for 10 min). Supernatant was collected and preserved frozen (−20 °C) for enzyme analysis.

Enzyme Assays

LDH (L-lactate NAD^+ oxidoreductase; E.C.1.1.1.27) was assayed using 0.1 M phosphate buffer (pH 7.5), 0.2 mM NADH solution in 0.1 M phosphate buffer. The reaction was initiated by adding 0.2 mM sodium pyruvate as the substrate and the OD was recorded at 340 nm [12]. A similar reaction mixture was used for the estimation of MDH (L-malate NAD^+ oxidoreductase; E.C.1.1.1.37) except for the substrate (1 mg oxaloacetate/mL of chilled triple distilled

water) [13]. ATPase (adenosine triphosphate phosphohydrolase, E.C.3.6.1.3) was assayed using a reaction mixture of 0.1 M Tris-HCl buffer (pH 7.8), 100 mM NaCl, 20 mM KCl, 3 mM MgCl₂, 5 mM ATP. The mixture was incubated for 15 min and the reaction was terminated by means of 10 % trichloroacetic acid [14]. Liberated phosphate was estimated at 660 nm [15]. Glucose was estimated by the method of Nelson and Somogyi [16].

At the end of 48 h, 50 recovering spawns from each tank (a total of 150 spawns/treatment groups) were subjected to temperatures higher than 37 °C in order to check for thermal tolerance and induction of cross protection. Their survivability rates were checked after exposure to 38, 39, 40, 41 and 42 °C.

Statistical Analysis

Enzymatic changes at different sampling stages for all treatments were investigated using one-way ANOVA. Post hoc test were carried out using Duncan's multiple comparison procedures, if they were significantly different. The statistical analysis was performed via SPSS 15.0 for windows. The level of significance employed was 5 % ($p < 0.05$).

Results and Discussion

Enzyme of Energy Metabolism: ATPase

ATPase demonstrated a sharp increase at 0 h after application of 1 h heat shock at 37 °C in group 1. A gradual decreasing trend was observed over the recovery period of 48 h in groups 1 and 2. Approximately 80 and 45 % reduction of ATPase activity was observed over 48 h of recovery period in groups 1 and 2, respectively. No significant difference was observed in the ATPase values at different recovery periods in the control (Table 1). Moreover, under each studied recovery period (0–48 h), group 2 (HS + Cl) registered significantly higher ATPase activities as compared to control. ATPase is a membrane bound enzyme and is responsible for the transport of ions through the membrane regulating Na⁺/K⁺ gradient along the cell membrane [9]. ATPase hydrolyses the high-energy phosphate (ATP) and utilizes that energy to maintain ionic gradient across the plasma membrane [17]. Akhtar et al. [18] reported increase in ATPase activity with increase in acclimation temperature.

In experiments 1 and 2, ATPase activity increased with 1 h stress and decreased gradually with different recovery hours. This is in congruence with the previous investigation in chinook salmon [19]. Thus the increase in ATPase activity after application of stress indicates the high need

Table 1 Enzymes of energy metabolism (ATPase), enzymes of metabolism (LDH and MDH) and glucose in *L. rohita* spawns was estimated throughout the recovery period of 48 h, after exposure to 1 h heat shock at 37 °C in group 1 (HS), 1 h heat shock 37 °C and chlorine (0.08 ppm) in group 2 (HS + Cl) and control in group 3 (C)

Parameters	Experimental groups	0 h	2 h	4 h	8 h	14 h	24 h	48 h
ATPase	HS	163.65 ± 5.23 ^{ab}	81.93 ± 6.71 ^{bb}	81.77 ± 6.52 ^{bb}	81.93 ± 6.71 ^{bb}	34.14 ± 42.23 ^{ab}	34.01 ± 3.09 ^{ab}	30.51 ± 5.21 ^{ab}
	HS + Cl	156.99 ± 39.25 ^{bb}	149.41 ± 45.12 ^{bc}	131.89 ± 6.97 ^{bc}	126.70 ± 0.28 ^{bc}	86.89 ± 7.06 ^{ab}	85.41 ± 72.61 ^{ab}	85.99 ± 38.07 ^{ab}
	C	47.45 ± 5.16 ^a	46.50 ± 2.12 ^a	47.50 ± 3.51 ^a	56.51 ± 3.51 ^a	53.00 ± 8.48 ^a	43.50 ± 2.12 ^a	44.50 ± 2.12 ^a
LDH	HS	1.77 ± 0.14 ^{bb}	0.46 ± 0.01 ^a	0.57 ± 0.13 ^a	0.54 ± 0.00 ^a	0.42 ± 0.04 ^a	0.38 ± 0.03 ^a	0.39 ± 0.04 ^a
	HS + Cl	0.64 ± 0.98 ^a	0.64 ± 0.13	0.60 ± 0.30	0.58 ± 0.13	0.54 ± 0.11	0.51 ± 0.084	0.50 ± 0.29
	C	0.61 ± 0.10 ^a	0.61 ± 0.11	0.64 ± 0.02	0.55 ± 0.21	0.63 ± 0.53	0.62 ± 0.26	0.60 ± 0.07
MDH	HS	2.36 ± 0.09 ^{db}	2.22 ± 0.15 ^{db}	1.40 ± 0.12 ^b	1.44 ± 0.12 ^b	1.79 ± 0.14 ^{cb}	1.44 ± 0.11 ^{bb}	0.91 ± 0.07 ^{ab}
	HS + Cl	2.21 ± 0.15 ^{cb}	1.58 ± 0.14 ^{ba}	1.55 ± 0.06 ^b	1.12 ± 0.07 ^a	1.10 ± 0.08 ^a	1.01 ± 0.03 ^{ab}	1.02 ± 0.03 ^{ab}
	C	1.19 ± 0.13 ^a	1.34 ± 0.13 ^b	1.60 ± 0.08	1.27 ± 0.92	1.34 ± 0.10 ^c	1.75 ± 0.35 ^b	1.44 ± 0.01 ^b
Glucose	HS	96.29 ± 5.30 ^{db}	75.03 ± 7.07 ^{cb}	64.40 ± 4.42 ^{bcB}	48.14 ± 15.02 ^b	42.51 ± 7.07 ^b	40.01 ± 14.14 ^b	33.63 ± 0.88 ^a
	HS + Cl	84.40 ± 0.88 ^{cb}	88.26 ± 3.53 ^{cb}	88.54 ± 9.72 ^{bb}	61.45 ± 10.61 ^b	61.52 ± 0.88 ^a	55.40 ± 0.88 ^a	41.40 ± 0.88 ^a
	C	39.01 ± 1.76 ^a	48.76 ± 17.68 ^a	36.50 ± 0.70 ^a	46.89 ± 0.88	43.76 ± 8.84	36.26 ± 0.00	37.51 ± 0.00

Values are expressed as mean ± SE ($n = 3$). Figures with different superscripts (a–d) in the same row indicate significant difference ($p < 0.05$) among different experimental groups (one-way ANOVA). Mean values ($n = 3$) in a column under each parameter bearing different superscripts (A–C) vary significantly ($p < 0.05$). Units: µg phosphorus released/mg protein/min at 37 °C (ATPase), µmol/mg protein/min (LDH and MDH), mg/dL (glucose)

for hydrolysis of ATP, a state where an increased supply of energy is needed. The decrease indicates recovery to approximately normal conditions. Control showed no significant difference indicating absence of stress.

Enzymes of Carbohydrate Metabolism

Lactate dehydrogenase (LDH)

LDH demonstrated an increased activity at 0 h after application of 1 h heat stress at 37 °C in group 1. A decreasing trend was observed over the recovery period of 48 h ($p < 0.05$). Approximately 77 % reduction of LDH activity was observed over 48 h of recovery period in group 1. However, no significant difference in LDH activity was found in group 2 and control at different recovery periods (Table 1). At 0 h of recovery, a significant difference was observed in LDH activities among different treatment groups, however, the activities were non-significant among the groups through 2–48 h of recovery period.

LDH (L-lactate NAD⁺ oxidoreductase; E.C.1.1.1.27) catalyses the terminal step in anaerobic glycolysis and, together with the other glycolytic enzymes, is located in the cytosol. The level of LDH activity and the functional properties of this enzyme reflect the capacity for anaerobic energy production and, thereby, the level of resistance to oxygen deficiency during hypoxia, vigorous exercise or thermal stress [20]. The elevated LDH activities indicated stress related tissue impairment along with metabolic changes associated with increased production of lactate.

LDH demonstrated an increased activity at 0 h after application of 1 h heat stress at 37 °C in group 1. The increase can be attributed to the production of preferred substrate (lactate) for gluconeogenesis [21, 22] or the hypoxic condition. Whole body LDH activity decreased significantly after 2 h of recovery and then gradually observed a decreasing trend. The gradual decrease shows recovery from hypoxic condition. Control showed no significant difference indicating the absence of stress or hypoxic condition.

Malate dehydrogenase (MDH)

MDH demonstrated an increased activity at 0 h after application of 1 h heat stress at 37 °C in group 1 as compared to control. A decreasing trend was observed over a recovery period of 48 h. A similar decreasing trend was observed in group 2 after exposure to chlorine and heat shock at 37 °C. Significant difference ($p < 0.05$) was observed among the values at different recovery periods in both the groups. The control group registered similar MDH activities (Table 1).

MDH is an enzyme of the TCA cycle, which oxidizes malate to oxaloacetate, using NAD⁺ as the electron acceptor and its activity is reported to increase under the influence of stress [7]. MDH activity also increases during gluconeogenesis. MDH demonstrated an increased activity at 0 h and a decreasing trend was observed over a recovery period of 48 h in groups 1 and 2. This may be due to increased gluconeogenesis when the spawns were under stress in order to meet the increased energetic demand and gradual recovery after the stressor is removed. MDH was increased at higher acclimation temperature in an experiment with *Cyprinus carpio* [8]. Control showed no significant difference indicating absence of stress.

Whole Body Glucose

A sharp increase in the body glucose level was observed at 0 h after the application of 1 h heat stress at 37 °C in group 1 as compared to control. A decreasing trend was observed over the recovery period of 48 h ($p < 0.05$). Similar trend was observed in group 2 after exposure to chlorine and heat shock at 37 °C ($p < 0.05$). However, the rate of decrease of glucose level over the recovery period of 48 h was rapid in group 1 as compared to the decrease in group 2. There was no significant trend observed among the treatments of the control group (Table 1). Approximately 3- and 2-fold decrease in the whole body glucose was evidenced over 48 h of recovery period in groups 1 and 2, respectively.

Ample literature exists on the rise of glucose level on application of various stressors like handling, transportation and thermal stress [7]. Studies have shown that catecholamines rise immediately after stress, and this transient increase results in rapid glycogen breakdown and consequently elevated plasma glucose concentration [23]. Therefore, measurement of blood sugar level is simple yet effective method to evaluate the effects of variety of stressors.

High whole body glucose was observed at 0 h sampling which decreases gradually up to 48 h. This shows that 1 h heat shock caused high glucose production to fulfill the energy requirement, which recovers gradually. Similar trend was observed in experiment 2 after exposure to chlorine and heat shock at 37 °C ($p < 0.05$). A gradual decrease was observed after 4 h of recovery in experiment 2; the reason is that glycogen is broken down into glucose in the early stages of stress [6, 24]. The rate of decrease of glucose levels over the recovery period of 48 h was rapid in experiment 1 as compared to the decrease in experiment 2 indicating that temperature and chlorine have more adverse effects on *L. rohita* spawns as compared to temperature alone. Control showed no significant difference indicating absence of stress.

Thermal Tolerance and Survivability

After completion of 48 h, spawns from group 1 (heat shock for one h at 37 °C) showed 100 % survival when exposed to 38, 39 and 40 °C but only 30 % survivability was observed when it was exposed to 41 °C. None survived at 42 °C. After completion of 48 h, spawns from group 2 (heat shock for 1 h in chlorinated water at 37 °C) showed 100 % survivability when exposed to 38 and 39 °C but only 40 % survivability was observed when it was exposed to 40 °C and 30 % survivability when it was exposed to 41 °C. None survived at 42 °C. Spawns exposed to higher temperatures after completion of 48 h from group 3 (control) showed no survivability when the spawns were exposed to 38 °C (Fig. 1).

Thermal tolerance is an endogenous mechanism for cells to withstand the thermal injury [25]. Thermal tolerance is influenced by acclimation temperature and thermal history of a species [7, 26]. Thermal tolerance can be achieved by induction of heat shock proteins (HSPs) in response to mild heat stress [27]. This finding was also supported by Das et al. [11]. Although HSPs have a relatively short half-life, their levels remain elevated in the whole organisms long after the stressor is removed, indicating a role in long term adaptation and increased stress tolerance leading to cross protection [28, 29]. In brown trout, 1 h heat shock was reported to induce HSP 70 in different tissues [30].

In the present study, CT_{max} for *L. rohita* spawns before commencing the experiment was found to be 38 °C. After recovery of 48 h, the spawns that were exposed to heat shock (37 °C) alone showed 100 % survival rates at 40 °C indicating increased thermal tolerance by 3 °C, whereas the spawns that were exposed to heat shock (37 °C) + chlorine, after 48 h recovery showed 100 %

survival rates at 39 °C. Hence the spawns exposed to mild heat shock alone have demonstrated the ability to tolerate higher temperature due to thermal tolerance than those exposed to heat shock and chlorine. This leads us to conclude that chlorine has more pronounced effect on *L. rohita* spawns and fails to develop the desired thermal tolerance that only heat shock alone provides. Hence higher thermal tolerance was observed when the spawns were exposed to heat shock (37 °C) alone.

Conclusion

Overall results indicate that *L. rohita* spawns demonstrate metabolic readjustments after being subjected to stress in order to cope up with energy demands of the cell. The increased enzyme activity gradually decreases or is inhibited during the recovery period. The difference in the rate of decrease between the two experiments—exposure to heat shock and heat shock + chlorine indicated that the spawns recovered faster in group 1 as compared to group 2, thus indicating that the dual effect of heat shock and chlorine does affect the spawns to a greater degree than when it is exposed to heat shock alone. Thus proving the hypothesis that the stressors when given in combination are more deleterious than the one given alone and the effect on the metabolism thus is affected more due to the dual application. Also greater thermal tolerance is developed in spawns when exposed to heat shock (37 °C) alone. The induced tolerance after exposure to heat shock alone may have improved recovery too. The exact mechanism of action by which dual stressors affect the specific metabolism remains elusive. Attention is invited from future researchers to investigate the same as the time taken for recovery indicates the extent of damage caused to the spawns due to stressors.

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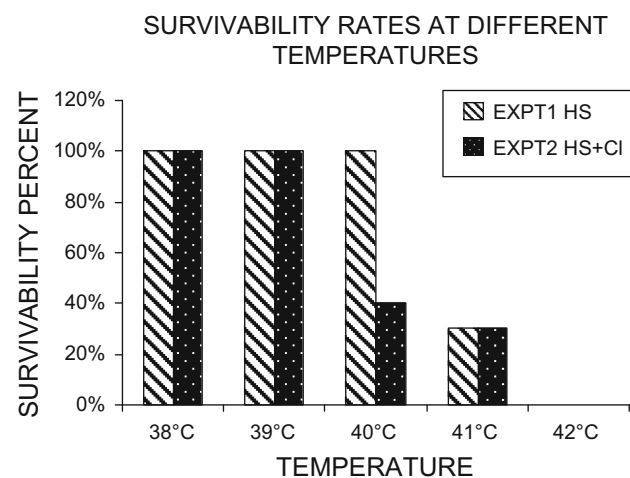


Fig. 1 The survivability percentage of *Labeo rohita* spawns on exposure to higher temperatures from the three groups after recovery of 48 h

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