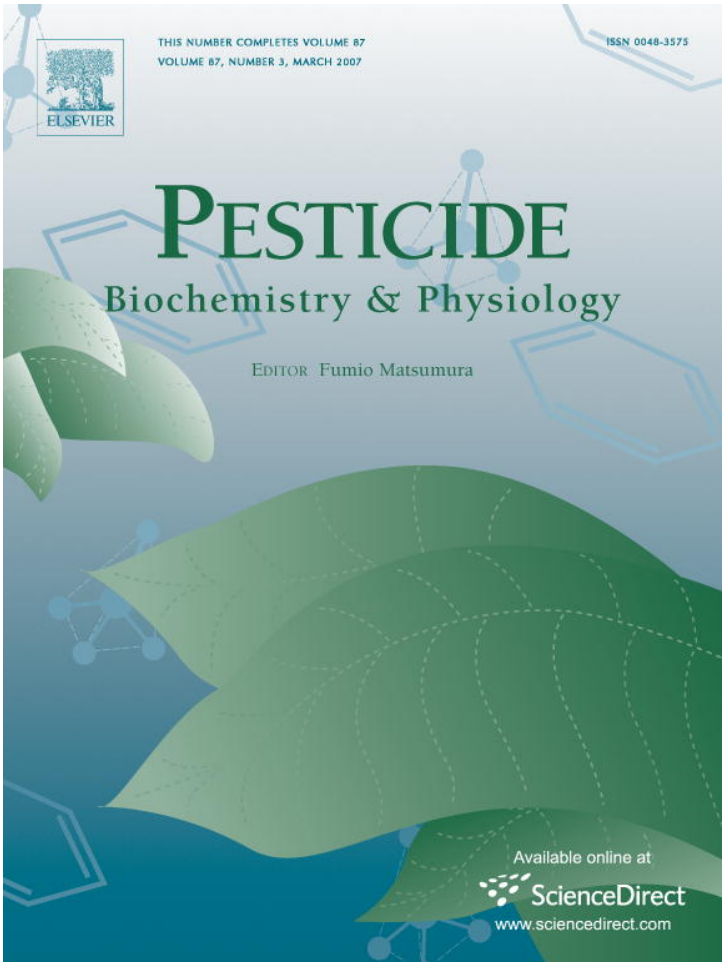

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# Persistent sub-lethal chlorine exposure elicits the temperature induced stress responses in *Cyprinus carpio* early fingerlings

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**Abstract**

Thermal effluents discharged through cooling systems of nuclear power plants often contain chlorine (used to control bio-fouling), which may affect the metabolic status of fishes. In order to evaluate the hypothesis, we tested the effect of high temperature and a persistent sub-lethal chlorine exposure on stress responses in *Cyprinus carpio* advanced fingerlings. Fishes were acclimated to four different temperatures (26, 31, 33, and 36 °C) and maintained for 30 days in two different groups. Subsequently, one of the groups was exposed to persistent chlorine (0.1 mg $L_{-1}$) for another 28 days and was compared with their respective temperature controls (without chlorine exposure). Sub-lethal doses of pollutants and increasing temperatures with in the tolerance range may not always register any morphological changes Therefore, we studied organ specific biochemical pathways viz. aspartate amino transferase, alanine amino transferase (enzymes of protein metabolism) in liver and muscle; fructose 1,6 diphosphatase (gluconeogenic pathway), in liver; pyruvate kinase, malate dehydrogenase, and lactate dehydrogenase (glycolytic pathway) in muscle; glucose-6-phosphate dehydrogenase (pentose phosphate pathway) in liver; alkaline phosphatase (phosphorus metabolism) in intestine, liver, and muscle; acetylcholine esterase (neurotransmitting enzyme) in brain, and adenosine triphosphate (for membrane transport) in gills at two different acclimation periods (14 and 28 days). The results indicate that *C. carpio* fingerlings demonstrated metabolic readjustments with increasing temperatures, in order to cope with energy demand of the cell. However, exposure to chlorine at higher temperatures affected protein metabolism, gluconeogenic pathway and subsequently glycolytic pathway, leading to an energy-limited condition. In addition, alteration of membrane transport and neurotransmission might be an early indication of cellular damage. Overall results indicate that persistent sub-lethal chlorine exposure elicits temperature induced stress response in *C. carpio* early fingerlings.


*Keywords:* *Cyprinus carpio*; Acclimation temperatures; Chlorine; Metabolic enzymes; Stress

## 1. Introduction

In India, electricity is mainly derived from thermal, hydroelectric, and nuclear power stations. Only about 30% of thermal energy generated from fuels is converted into electricity and the remaining heat is released into the environment through water bodies. For the efficient operation of a nuclear power plant, uninterrupted supply of cooling water into the condensers is pre-requisite [1]. Nuclear power plants require, on an average, about 3 $m^3$ cooling water per minute per megawatt (MW) of electricity produced [2]. The dissipation of heat through the plant cooling water systems may affect growth, development, and

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distribution of flora and fauna in the surroundings, which is an important area of concern among the plant operators and environmentalists. Apart from increased temperature, the discharges often contain chemical stress factors in the form of biocides (e.g., chlorine) used for biofouling control [3]. Thus, condenser effluents from thermal power plants have the potential to impart thermal and chemical stress and, therefore, may pose environmental problems to the receiving water body [4]. In addition, increasing greenhouse gases in the atmosphere creates global warming and climatic changes over the years. The United States National Research Council proposed that the global mean temperature may increase by 1.5–4.5 °C in the next half century [5]. Thus, potential effect of thermal effluents discharge from power plants in addition to the increasing temperature due to global warming and climatic changes makes it vital to define the impact of temperature on fishes. In addition, the potential impact of chlorine in thermal effluents on fish physiology is not yet clearly understood.

Common carp (*Cyprinus carpio* L.) is the oldest cultured and the most domesticated fish species of the world [6]. *C. carpio* culture is well suited in temperate as well as tropical countries of the globe, as they can tolerate a wide range of environmental conditions and endure relatively poor water quality. Carps can survive extreme temperatures (4–39 °C) and low dissolved oxygen for several days [7]. In northern hemisphere, they are reported to tolerate 0–30 °C. However, the optimal temperature for carp growth from studies has been found to be around 25 °C [8]. Against this background, the present study was undertaken to assess the stress effect of increasing temperature combined with sub-lethal (0.1 mg $L^{-1}$) level of chlorine, using *C. carpio* early fingerlings as a biomarker species. The acclimation temperatures chosen were 26, 31, 33, and 36 °C since it is in the range of preferred temperature of common carp, *C. carpio* [9] Indian Major Carps [10] Chlorine levels in the immediate vicinity of thermal power plants (near discharge canal) is about 0.1 mg $L^{-1}$ (personal communication from power plant operators).

Thermal acclimation process and their limits have been determined by enzyme activities at different temperature regimes [11]. We tested a few metabolic enzymes to describe the cellular physiology of the fish after exposure to high temperature and chlorine. Fishes utilize protein and lipid sources than carbohydrate for meeting their energy requirement [12]. Therefore, gluconeogenic pathway and protein metabolism, assumes importance in fishes under stress [13]. We analysed transaminases (alanine amino transferase, ALT and aspartate amino transferase, AST), enzymes of gluconeogenic pathway (fructose-1,6-diphosphatase, FDPase), glycolytic pathway (pyruvate kinase, PK malate dehydrogenase, MDH, and lactate dehydrogenase, LDH), pentose phosphate pathway (Glucose 6 phosphate dehydrogenase, G6PDH), phosphorus metabolism (alkaline phosphatase, ALP), neurotransmission (acetylcholine esterase, AchE), and membrane transport (adenosine triphosphatase, ATPase). ALT and AST were tested because it produces TCA cycle intermediates by transamination, which is used as the preferred substrate for gluconeogenesis [14,15]. PK is an allosteric enzyme, which act as a negative control of glycolysis, the main pathway for metabolic energy. The presence of FDPase in the liver, may act as a forward stimulator of PK. LDH, the terminal enzyme of glycolytic pathway, is responsible for reversible conversion of pyruvate to lactate and is present in most of the tissues. Under stressed condition, anaerobic pathway is activated and high levels of lactate are being produced by inter-conversion of pyruvate in the cell. Similarly, MDH (a sulfhydryl enzyme), which is involved in reversible conversion of L-malate and oxaloacetate. MDH activity is reported to increase under the influence of stress [16]. ALP, a zinc-containing metallo-enzyme, which plays an important role in metabolism of phosphorus in the body, which demonstrates stressor specific responses. Enzyme of neurotransmission, AchE is widely distributed in nervous tissue, stored and released from the synaptic vesicles, which helps in the depolarization of adjacent neuron and thus passes nerve signals. AchE is rapidly hydrolysed by choline esterase [17] under normal physiological conditions. AchE is said to be inhibited under the influence of xenobiotics, which affects the neurotransmission. ATPase is a membrane bound enzyme responsible for the transport of ions through the membrane and regulates $Na^+/K^+$ gradient along the cell membrane [18] and is considered affected under stress. From our earlier observations in experiments in fish (data unpublished), shellfish [19], these enzymes showed organ specific activity. Therefore, we tested ALT and AST in liver and muscle; FDPase in liver; PK, MDH, and LDH in muscle; G6PDH in liver; ALP in intestine, liver, and muscle; AchE in brain, and ATPase in gills. This organ specific metabolic profile may improve our current understanding on the impact of prolonged exposure to temperature and chlorine on the metabolic stress responses in *C. carpio* early fingerlings.

## 2. Materials and methods

### 2.1. Experimental fishes

*Cyprinus carpio* fingerlings (mean ± SE : 5.3 g ± 0.2) were brought in separate aerated open containers from Khopoli fish seed farm, Government of Maharashtra to wet laboratory, Central Institute of Fisheries Education, Mumbai and were acclimated for 30 days to laboratory conditions (30 °C). During this period, fishes were fed with supplementary feed (25% crude protein) before being subjected to acclimatory studies.

### 2.2. Acclimation of experimental fishes

A total of 144 fishes were distributed in eight different treatments in thermostatic aquaria (175 L water capacity) at the rate 1 °C/day from laboratory temperature (26 °C) to reach experimental acclimation temperatures (26, 31, 33,

and 36 °C) each with (18 fishes per thermostatic aquaria of 175 L water capacity, sensitivity ± 0.2 °C) and maintained for another 30 days prior to the experiment. From one of our previous investigations on the effect of acclimation temperatures on thermal tolerance and oxygen consumption suggested that Indian major carps were completely acclimated to test temperatures in 30 days [10]. In another study, Sheepshead minnow, *Cyprinodon variegatus* was completely acclimated to laboratory conditions after 30 days [20]. Therefore, we assumed that the test fishes for the present investigation are successfully acclimated to test temperatures at the end of 30 days acclimation. Further, a uniform level of chlorine (0.1 mg $L^{-1}$) was maintained in one treatment group at all the four-acclimation temperatures throughout the experimental phase (which was monitored thrice daily). The evaporation rate was calibrated and the required amount of chlorine was added to rearing water in order to maintain uniform chlorine levels. Other group without chlorine served as respective control at each acclimation temperatures.

### 2.3. Feed and feeding

Formulated pelleted feeds (30% crude protein) from various ingredients (fish meal, soybean meal, wheat bran, and rice bran as basal diet and protein source) were used for feeding the fishes during the entire experimental phase. Fishes were fed *ad libitum* on a daily basis. Siphoning of waste feed and faecal materials were done on the next day before dispensing the feed. Water exchange was carried out (using temperature adjusted chlorine free freshwater up to 50%) every alternate day. Fishes were starved for a day prior to sampling (14 and 28 days).

### 2.4. Chlorine dosage and analysis

Chlorine level was monitored every 8 h prior to the starting of the experiment, calibrating the evaporation rate with increasing acclimation temperatures and the required dose of chlorine was determined. During the experiment, chlorine was supplemented at every 8-h interval as per the evaporation rate to maintain the level of chlorine in experimental tanks at 0.1 mg $L^{-1}$. For the addition of chlorine a hypochloride solution, HPLC grade was used. Chlorine levels were continuously monitored by using Spectroquant Chlorine test kit (1. 00 599. 0001, E Merck, Germany) with accuracy (0.01 mg $L^{-1}$).

### 2.5. Sample preparation

Fishes were sampled at two different acclimation periods 14 days (considered short term acclimation) and 28 days (long term acclimation) from all treatments (26, 31, 33, and 36 °C) with and without chlorine (control) after being anaesthetized with Cifecalm (200 μL $L^{-1}$). Cifecalm is an herbal anesthetic formulation containing natural alcoholic extracts of *Eugenia caryophyllata,* and *Mentha arvensis* (developed by Central Institute of Fisheries Education, Mumbai). Pre-weighed liver (0.1 g), muscle and gill (0.5 g), intestine (0.2 g), and brain (0.1 g) was homogenized in chilled sucrose solution (0.25 M) by mechanical tissue homogenizer and was centrifuged (5000 rpm at 4 °C for 10 min). Supernatant was collected and preserved frozen (−20 °C) for enzymatic studies.

### 2.6. Metabolic enzyme assays

Alanine amino transferase (L-alanine 2 oxaloglutarate aminotransferase; E.C.2.6.1.2) 0.2 M D, L-alanine and 2 mM α-ketoglutarate in 0.05 M phosphate buffer (pH 7.4) as the substrate and aspartate amino transferase (L-aspartate: 2 oxaloglutarate aminotransferase, E.C.2.6.1.1) using 0.2 M D L- aspartic acid and 20 mM α-ketoglutarate in 0.05 M phosphate buffer (pH 7.4) as the substrate and both were estimated at optical density (OD) of 540 nm [21]. fructose-1, 6-diphosphatase (D-FDP-1-Phosphohydrolase; E.C. 3.1.3.11) was estimated using 50 mM borate buffer (pH 9.5), 0.05 M fructose-1,6-diphosphate (pH 7–7.3) and 0.5 M $MgSO_4$ [22]. Adenosine triphosphatase (adenosine triphosphate phosphohydrolase, E.C. 3.6.1.3) using a reaction mixture of 0.1 M Tris–HCl buffer (pH 7.8), 100 mM NaCl, 20 mM KCl, 3 mM $MgCl_2$, 5 mM ATP. The mixture was incubated for 15 min and the reaction was terminated by means of 10% trichloroacetic acid [23]. Phosphate liberated in both cases was estimated at OD of 660 nm [24]. Pyruvate kinase (pyruvate kinase, E.C. 2.7.1.40) using a mixture of 0.05 M imidazole–HCl buffer (pH 7.6) containing 0.12 M potassium chloride and 0.062 M magnesium sulfate, 45 mM adenosine diphosphate, 45 mM phosphoenolpyruvate, 6.6 mM NADH, lactate dehydrogenase (1300 U/ml) in the imidazole buffer were well mixed and incubated for 4–5 min. Addition of diluted enzyme initiated the reaction and the decrease in ODs at 340 nm was recorded for 3 min [25]. Glucose-6-phosphate dehydrogenase (α-D-glucose-6 phosphate: NADP oxidoreductase; E.C.1.1.1.49) using a reaction mixture of 0.1 M Tris buffer (pH. 7.8), 2.7 mM NADP, 0.02 M glucose-6-phosphate (substrate). The rate of reaction was recorded at 340 nm at 15 s intervals for 3 min [26]. Lactate dehydrogenase (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 OD was recorded at 340 nm [27]. A similar reaction mixture was used for the estimation of malate dehydrogenase (L-malate: $NAD^+$ oxidoreductase; E.C.1.1.1.37) except for the substrate (1 mg oxaloacetate/ml of chilled triple distilled water) [28]. Alkaline phosphatase (Orthophosphoric monoester phosphohydrolase, E.C.3.1.3.1) using an assay mixture comprised of 0.2 M bicarbonate buffers, 0.1 M $MgCl_2$ and 0.1 M *para*-nitrophenyl phosphate. The reaction mixture was incubated at 37 °C (15 min) and the reaction was arrested by 0.1 N NaOH and read the absorbance at 410 nm [29]. Acetylcholine esterase (acetyl hydroxylase, E.C.3.1.1.7) was assayed using a

mixture of M/15 phosphate buffer (pH 7.2), 0.004 M acetylcholine (pH 4.0) and a substrate buffer mixture (1/10 dilution), incubated for 30 min at 37 °C. Alkaline hydroxylamine solution was used to terminate the reaction and HCl (2:1) was added. The colour was developed by addition of 10% $FeCl_3$ and absorbance was read at 540 nm after thorough mixing [30]. Total protein content was analysed from the supernatant [31] for calculating enzyme activities. All the colorimetric assays were carried out using UV–ViS spectrophotometer (E-Merck, Germany).

### 2.7. Statistical analyses

Enzymatic changes at different acclimation temperatures (26, 31, 33, and 36 °C) with and without chlorine were investigated by using one-way ANOVA. Post hoc test were carried out using Duncan's multiple comparison procedures, if they were significantly different. Student's *t* test was performed to assess the significance between the two-acclimation phases. All the statistical analyses were performed via SPSS 12.0 for Windows.

## 3. Results

### 3.1. Enzymes of protein metabolism

#### 3.1.1. Aspartate amino transferase (AST)

In liver, AST activity increased with increasing acclimation temperatures in both control and chlorine treatments ($p < 0.05$) after 14 days acclimation. However, lower levels of AST activity are evident in response to chlorine. After 28 days acclimation, AST activity appeared stabilized in liver at higher acclimation temperatures. In case of muscle, AST activity increased with increasing acclimation temperatures in both control and chlorine treatments ($p < 0.05$) at both acclimation periods (14 and 28 days). AST activity showed a decreasing trend with increasing acclimation periods (Table 1).

#### 3.1.2. Alanine amino transferase (ALT)

In liver, ALT activity increased with increasing acclimation temperatures after 14 days acclimation in both control and chlorine treated groups. However, *t* test comparison between control and chlorine treatments reveals a decreasing trend in latter over the former treatments, irrespective of acclimation temperatures. However, after 28 days acclimation, ALT activity appears stabilized at higher acclimation temperatures. In muscle, ALT activity increased with increasing acclimation temperatures after 14-day acclimation. However, after 28 days, ALT activity was significantly different in both control and chlorine treated groups ($p < 0.05$) although no consistent trend was observed (Table 1).

### 3.2. Gluconeogenic enzymes

#### 3.2.1. Fructose 1,6 diphosphatase (FDPase)

After 14 and 28 days acclimation, FDPase activity in liver increased significantly ($p < 0.05$) with increasing temperatures in both control and chlorine treated groups except at 36 °C (after 28 days). However, after 14 days acclimation, FDPase activity was inhibited in chlorine treated groups as compared to control (Table 1).

### 3.3. Glycolytic enzymes

#### 3.3.1. Pyruvate kinase (PK)

After 14 and 28 days acclimation, PK activity in liver increased significantly ($p < 0.05$) with increasing temperatures in both control and chlorine treated groups. Due to chlorine treatment, PK was comparatively higher over control after 14 days acclimation. However, after 28 days acclimation, PK activity was inhibited in chlorine treated groups as compared to control (Table 1).

#### 3.3.2. Malatate dehydrogenase (MDH)

MDH demonstrated an increase in activity with increasing temperatures in both chlorine treated groups and their respective temperature controls ($p < 0.05$). MDH activity appeared stabilized with increasing acclimation duration (at the end of 28 days) (Table 1).

#### 3.3.3. Lactate dehydrogenase (LDH)

LDH demonstrated an increase in activity with increasing temperatures in control groups except at 31 °C ($p < 0.05$). In chlorine treated groups, LDH activity was found inhibited. A stabilizing trend was observed with increasing acclimation duration (at the end of 28 days) on both control and chlorine treated groups (Table 1).

### 3.4. Enzymes of pentose phosphate pathway

#### 3.4.1. Glucose-6-phosphate dehydrogenase (G6PDH)

After 14 days acclimation, G6PDH activity in liver decreased with increasing acclimation temperatures. After 14 days acclimation, G6PDH activity was higher in chlorine treated groups, over control. However, after 28 days acclimation, the impact of chlorine on *C. carpio* was evident from lower G6PDH activity in chlorine treated groups as compared to control ($p < 0.05$) (Table 2).

### 3.5. Enzymes of phosphate metabolism

#### 3.5.1. Alkaline phosphatase (ALP)

After 14 days acclimation, ALP activity in intestine increased significantly ($p < 0.05$) with increasing temperatures in both control and chlorine treated groups. Higher ALP activity was observed with increasing acclimation temperatures (31, 33, and 36 °C) after 28 days acclimation. However, chlorine treatments found to inhibit ALP activity in *C. carpio.* In liver, ALP activity increased significantly with increasing temperatures in both control and chlorine treated groups at the end of both acclimation periods. However, chlorine treatment found to inhibit ALP activity in *C. carpio.* In muscle, ALP activity demonstrated no definite trend with increasing acclimation temperatures in both

Table 1
Enzymes of protein metabolism (alanine amino ransferase, ALT and aspartate amino transferase, AST), gluconeogenic pathway (fructose 1, 6 diphoshphatase, FDPase), glycolytic pathway (pyruvate kinase and malate dehyrogenase, MDH) and anaerobic pathway (lactate dehydrogenase, LDH) in *C.capio* early fingerlings after exposure to chlorine at four different temperatures (26, 31, 33, 36 °C) for 14 and 28 days

| Enzyme | Acclimation Period | Temperature (°C) | | | | Temperature (°C) + 0.1 mg $L^{-1}$ Chlorine | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | 26 | 31 | 33 | 36 | 26 | 31 | 33 | 36 |
| AST (liver) | 14 days | $105 \pm 3.7^{c,*}$ | $107.66 \pm 1.8^{c,*}$ | $115.83 \pm 14.8^{f}$ | $119.41 \pm 2.5^{f,*}$ | $66.24 \pm 1.6^{a,*}$ | $78.45 \pm 2.8^{b,*}$ | $86.17 \pm 2.8^{c,*}$ | $91.76 \pm 4.1^{d,*}$ |
| | 28 days | $177 \pm 11.7^{e}$ | $134.02 \pm 4.2^{d}$ | $110.46 \pm 4.9^{b}$ | $86.81 \pm 1.5^{a}$ | $143.78 \pm 4.9^{d}$ | $122.72 \pm 6.9^{c}$ | $139.15 \pm 1.3^{d}$ | $136.03 \pm 3.89^{d}$ |
| AST (muscle) | 14 days | $66.25 \pm 0.62^{b,*}$ | $60.88 \pm 0.75^{a,*}$ | $73.48 \pm 2.67^{d,*}$ | $83.00 \pm 1.21^{f,*}$ | $60.05 \pm 1.04^{a}$ | $71.87 \pm 0.82^{c,*}$ | $76.83 \pm 1.08^{e,**}$ | $85.50 \pm 0.32^{g}$ |
| | 28 days | $75.66 \pm 1.18^{b}$ | $95.60 \pm 2.95^{d}$ | $108.79 \pm 2.01^{e}$ | $118.16 \pm 3.10^{f}$ | $58.04 \pm 1.66^{a}$ | $61.37 \pm 1.62^{a}$ | $83.86 \pm 2.30^{c}$ | $83.41 \pm 2.85^{c}$ |
| ALT (liver) | 14 days | $21.58 \pm 1.25^{a,*}$ | $28.59 \pm 1.16^{b,**}$ | $43.18 \pm 0.19^{e}$ | $45.20 \pm 0.97^{f,*}$ | $20.36 \pm 0.70^{a,*}$ | $22.36 \pm 0.67^{a,*}$ | $33.15 \pm 1.02^{c,***}$ | $38.31 \pm 1.65^{d,*}$ |
| | 28 days | $60.91 \pm 7.72^{e}$ | $51.56 \pm 7.66^{cd}$ | $40.48 \pm 2.12^{b}$ | $70.13 \pm 2.32_{f}$ | $45.75 \pm 1.94^{b}$ | $54.21 \pm 3.04^{cde}$ | $28.49 \pm 3.08^{a}$ | $61.74 \pm 1.50^{e}$ |
| ALT (muscle) | 14 days | $9.89 \pm 0.90^{b,*}$ | $6.57 \pm 0.22^{a,*}$ | $10.80 \pm 0.58^{b,*}$ | $18.04 \pm 1.52^{c,*}$ | $10.58 \pm 0.97^{b,*}$ | $11.17 \pm 1.15^{b,**}$ | $17.84 \pm 1.01^{c,***}$ | $24.80 \pm 1.20^{d}$ |
| | 28 days | $22.28 \pm 1.08b^{c}$ | $20.88 \pm 0.97^{ab}$ | $22.84 \pm 0.80^{bcd}$ | $26.25 \pm 0.66^{e}$ | $18.83 \pm 1.58^{a}$ | $18.30 \pm 1.28^{a}$ | $25.04 \pm 2.41^{cde}$ | $25.91 \pm 2.24^{de}$ |
| FDPase (liver) | 14 days | $23.70 \pm 1.23^{a}$ | $56.23 \pm 8.35^{c}$ | $65.49 \pm 9.18^{c}$ | $135.61 \pm 11.22^{d,*}$ | $20.91 \pm 1.69^{a}$ | $33.52 \pm 2.75^{ab,*}$ | $48.03 \pm 3.75^{bc,**}$ | $157.61 \pm 11.53^{e,*}$ |
| | 28 days | $24.87 \pm 2.26^{a}$ | $59.46 \pm 2.63^{cd}$ | $51.99 \pm 4.12^{cd}$ | $15.76 \pm 1.63^{ab}$ | $28.90 \pm 2.34^{b}$ | $50.18 \pm 2.85^{c}$ | $75.49 \pm 5.83^{e}$ | $61.00 \pm 4.86^{d}$ |
| Pyruvate kinase (liver) | 14 days | $0.403 \pm 0.016^{a}$ | $0.491 \pm 0.009^{b,*}$ | $0.823 \pm 0.014^{e,*}$ | $1.149 \pm 0.021^{d,**}$ | $1.340 \pm 0.069^{e,*}$ | $1.353 \pm 0.031^{e,*}$ | $1.548 \pm 0.015^{f,*}$ | $1.904 \pm 0.030^{g,*}$ |
| | 28 days | $0.484 \pm 0.039^{b}$ | $0.595 \pm 0.019^{c}$ | $0.979 \pm 0.011^{d}$ | $1.19 \pm 0.05^{f}$ | $0.315 \pm 0.013^{a}$ | $0.463 \pm 0.026^{b}$ | $0.619 \pm 0.051^{c}$ | $1.108 \pm 0.027^{e}$ |
| MDH (muscle) | 14 days | $11.74 \pm 0.19^{d,*}$ | $13.22 \pm 0.44^{e,*}$ | $13.74 \pm 0.26^{f,*}$ | $14.73 \pm 0.17^{g,*}$ | $9.89 \pm 0.03^{a,*}$ | $10.91 \pm 0.07^{b,*}$ | $11.12 \pm 0.13^{b,*}$ | $11.41 \pm 0.11^{c,**}$ |
| | 28 days | $5.52 \pm 0.21^{a}$ | $5.27 \pm 0.23^{a}$ | $6.91 \pm 0.09^{bc}$ | $7.59 \pm 0.05^{c}$ | $6.47 \pm 0.14^{b}$ | $5.44 \pm 0.39^{a}$ | $7.14 \pm 0.61^{bc}$ | $9.30 \pm 0.70^{d}$ |
| LDH (muscle) | 14 days | $15.37 \pm 0.24^{f,*}$ | $14.96 \pm 0.03^{d,*}$ | $15.28 \pm 0.21^{d,*}$ | $17.97 \pm 0.44^{e,*}$ | $14.27 \pm 0.02^{c,*}$ | $11.85 \pm 0.13^{a,*}$ | $12.40 \pm 0.19^{b,*}$ | $12.61 \pm 0.03^{b,*}$ |
| | 28 days | $8.47 \pm 0.21^{d}$ | $7.78 \pm 0.15^{c}$ | $10.01 \pm 0.13^{e}$ | $10.89 \pm 0.22^{f}$ | $6.21 \pm 0.22^{b}$ | $5.12 \pm 0.13^{a}$ | $7.58 \pm 0.32^{c}$ | $10.06 \pm 0.22^{e}$ |

Values are expressed as mean ± SE ($n = 6$). Different superscripts (a, b, c, d, e, f, g, h) in the same row indicate significant difference amongst different treatments (Duncan's multiple range test, $\alpha = 0.05$). Different superscripts (*$p < 0.001$, **$p < 0.01$, ***$p < 0.05$) in the same column indicate significance between two different acclimation periods (Student's *t*-test). Units: nanomole oxaloacetate formed/mg protein/min at 37 °C (AST), nanomoles pyruvate formed/mg protein/min at 37 °C (ALT), microgram phosphorus released/mg protein/min at 37 °C (FDPase), micromoles/mg protein/min (Pyruvate kinase), micromoles/mg protein/min (LDH and MDH).

Table 2
Enzymes of pentose phosphate pathway (glucose-6-phosphate dehydrogenase, G6PDH), phosphorus metabolism (alkaline phosphatase, ALP), enzymes of neurotransmission (acetylcholine esterase, AchE) enzyme related to membrane transport (ATPase) in *C.capio* early fingerlings after exposure to chlorine at four different temperatures (26, 31, 33, 36 °C) for 14 and 28 days

| Enzyme | Acclimation Period | Temperature (°C) | | | | Temperature (°C) + 0.1 mg $L^{-1}$ Chlorine | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | 26 | 31 | 33 | 36 | 26 | 31 | 33 | 36 |
| G6PDH (liver) | 14 days | $0.393 \pm 0.010^{c,***}$ | $0.289 \pm 0.001^{b,*}$ | $0.278 \pm 0.008^{b,*}$ | $0.234 \pm 0.004^{a,*}$ | $0.573 \pm 0.005^{e,*}$ | $0.715 \pm 0.006^{f,*}$ | $0.425 \pm 0.198^{d,*}$ | $0.378 \pm 0.02^{c}$ |
| | 28 days | $0.342 \pm 0.029^{d}$ | $0.401 \pm 0.010^{ef}$ | $0.393 \pm 0.007^{e}$ | $0.423 \pm 0.006^{f}$ | $0.217 \pm 0.005^{b}$ | $0.106 \pm 0.007^{a}$ | $0.309 \pm 0.012^{c}$ | $0.390 \pm 0.013^{e}$ |
| ALP (intestine) | 14 days | $92.03 \pm 2.06^{b*}$ | $122.02 \pm 4.83^{c,*}$ | $236.58 \pm 2.35^{f,*}$ | $262.90 \pm 1.92^{g,*}$ | $89.93 \pm 3.02^{a,*}$ | $99.14 \pm 3.08^{b}$ | $157.16 \pm 4.07^{d,*}$ | $173.79 \pm 11.3^{e,*}$ |
| | 28 days | $268.34 \pm 3.32^{g}$ | $157.33 \pm 6.24^{d}$ | $165.96 \pm 4.63^{e}$ | $191.15 \pm 4.18^{f}$ | $152.56 \pm 3.43^{d}$ | $90.95 \pm 5.59^{a}$ | $108.95 \pm 5.22^{b}$ | $134.74 \pm 4.38^{c}$ |
| ALP (liver) | 14 days | $14.77 \pm 0.88^{b*}$ | $13.51 \pm 0.99^{ab,*}$ | $21.88 \pm 0.23^{c,*}$ | $31.85 \pm 1.19^{f,*}$ | $14.87 \pm 0.73^{b,*}$ | $12.72 \pm 0.33^{a,*}$ | $17.73 \pm 0.43^{c,*}$ | $20.21 \pm 1.94^{d,*}$ |
| | 28 days | $26.69 \pm 1.18^{ab}$ | $29.99 \pm 1.59^{c}$ | $39.65 \pm 1.34^{de}$ | $41.77 \pm 1.14^{e}$ | $26.19 \pm 1.9^{a}$ | $28.74 \pm 1.68^{bc}$ | $38.37 \pm 2.68^{d}$ | $41.30 \pm 1.65^{e}$ |
| ALP (muscle) | 14 days | $27.96 \pm 0.13^{d,*}$ | $21.08 \pm 1.04^{bc,*}$ | $18.88 \pm 2.06^{b}$ | $15.00 \pm 1.28^{a,*}$ | $28.78 \pm 2.21^{d}$ | $14.35 \pm 1.12^{a,***}$ | $23.02 \pm 2.57^{c}$ | $19.94 \pm 3.38^{bc,**}$ |
| | 28 days | $31.52 \pm 1.22^{d}$ | $32.69 \pm 3.0^{d}$ | $22.11 \pm 2.18^{b}$ | $11.9 \pm 0.74^{a}$ | $26.71 \pm 1.79^{c}$ | $21.06 \pm 3.57^{b}$ | $23.87 \pm 1.64^{bc}$ | $32.79 \pm 3.74^{d}$ |
| AChE (brain) | 14 days | $75.44 \pm 1.98^{f,*}$ | $62.82 \pm 9.13^{e,*}$ | $50.38 \pm 2.87^{c,*}$ | $57.64 \pm 3.05^{de,*}$ | $48.71 \pm 1.24^{c,*}$ | $36.15 \pm 1.94^{a,*}$ | $18.67 \pm 1.07^{b,*}$ | $53.68 \pm 1.02^{cd,*}$ |
| | 28 days | $31.88 \pm 1.06^{e}$ | $30.47 \pm 1.25^{d}$ | $27.23 \pm 1.25^{c}$ | $26.82 \pm 1.14^{c}$ | $32.64 \pm 1.14^{f}$ | $30.98 \pm 1.15^{d}$ | $26.06 \pm 1.08^{b}$ | $24.48 \pm 1.13^{a}$ |
| ATPase (gill) | 14 days | $1.06 \pm 0.01^{a,*}$ | $1.13 \pm 0.02^{a,*}$ | $1.56 \pm .01^{b,*}$ | $1.70 \pm 0.01^{c,*}$ | $2.29 \pm 0.02^{d,*}$ | $2.30 \pm 0.02^{d,*}$ | $4.20 \pm 0.02^{e,*}$ | $5.81 \pm 0.08^{f,*}$ |
| | 28 days | $0.530 \pm 0.006^{b}$ | $0.455 \pm 0.063^{a}$ | $0.651 \pm 0.005^{c,d}$ | $0.686 \pm 0.001^{d}$ | $0.641 \pm 0.008^{cd}$ | $0.529 \pm 0.007^{b}$ | $0.652 \pm 0.010^{cd}$ | $0.778 \pm 0.014^{e}$ |

Values are expressed as mean ± SE ($n = 6$). Different superscripts (a, b, c, d, e, f, g, h) in the same row indicate significant difference amongst different treatments (Duncan's multiple range test, $\alpha = 0.05$). Different superscripts ($^{*}p < 0.001$, $^{**}p < 0.01$, $^{***}p < 0.05$) in the same column indicate significance between two different acclimation periods (Student's *t*-test). Units: Δ 0.01 optical density/mg protein/minute (G6PDH), nanomoles *p*-nitrophenol released/mg protein/minute at 37 °C (ALP), micromoles acetylcholine hydrolyzed/mg protein/min at 37 °C (AchE), microgram phosphorus released/mg protein/minute at 37 °C (ATPase).

control and chlorine treated groups at the end of 14 days. However, after 28 days acclimation, ALP activity increased significantly ($p < 0.05$) with increasing temperatures in both control and chlorine treated groups. (Table 2).

### 3.6. Enzymes of neurotransmission

#### 3.6.1. Acetylcholine esterase (AchE)

A decrease in AchE activity in brain was observed with increasing temperatures, 26 to 36 °C ($p < 0.05$). Presence of chlorine found to augment the inhibition AChE activity brain of *C. carpio* over their respective controls (Table 2).

### 3.7. Enzymes of membrane transport

#### 3.7.1. Adenosine triphosphatase (ATPase)

T test comparison revealed significantly higher ATPase in gills of *C.carpio* in chlorine treated groups at the end of 28 days over 14 days ($p < 0.05$). However, lower ATPase activity was recorded at the end of 28days as compared to 14 days in control groups ($p < 0.05$) (Table 2).

## 4. Discussion

Temperature is one of the most important abiotic factors, which influences all biochemical reactions and therefore has a great impact on the physiology and biochemistry of ectothermic organisms. Acclimation to increasing temperatures in different fishes viz., *Rhinomugil corsula* H. [32] and *Salvelinus alpinus* L. [33] is accompanied by changes in the relative activities of glycolytic and mitochondrial enzymes [34]. Investigations on the impact of intermittent chlorine exposure to simulate steam electric generating plants on five different fish fingerlings indicated that temperature played relatively little effect on the lethal concentration of either free chlorine or monochlorine [35]. On the contrary, another finding [36] on combined effects of chlorine, increased temperature, and time of exposure on early life stages of the mummichog (*Fundulus heteroclitus*) indicates that, change in temperature ($\Delta T$) alone was the most important variable causing death to three of four embryonic stages. However, a review of these reports [37] concluded that total residual chlorine, $\Delta T$ and exposure time has been called upon for further investigation. One of our earlier reports indicate that increasing acclimation temperature plays significant role in embryonic development of *Labeo rohita* [38] *Macrobrachium rosenbergii* [39] embryonic stages. In the present experiment, acclimatory responses to temperature and chlorine have been correlated with metabolic enzymes, to derive a more realistic picture on physiological status of *C. carpio* early fingerlings.

### 4.1. Effect on protein metabolism

High temperature creates higher free amino acid mobilization, as higher enzyme activities of transaminases; ALT and AST [40] were observed in *C. carpio* with increasing

acclimation temperatures, which in turn might have produced glucose to cope up with the stress, in the process of higher gluconeogenesis. Similar observation was recorded in Tilapia after being exposed to confinement stress [41]. In present study, ALT and AST activity in liver were inhibited with increasing acclimation temperatures in presence of chlorine, which might be due to the inhibition of *de novo* synthesis of protein. Similar pattern of inhibition of transaminase enzymes was observed in the liver of *C. carpio* in the chlorine treated groups during the first 14 days of the acclimation phase. Similar finding of cadmium in fish [42] indicate a significant reduction in AST and ALT levels in liver, while there was an increase in serum and suggested hepatocytes alters cell membrane structure. Both AST and ALT activity in the muscle was found higher than the control group suggesting that the keto acids might be directly entering into the Krebs cycle for energy yielding process.

### 4.2. Effect on gluconeogenic pathway

An increase in FDPase in reponse to increasing temperatures at the end of two different acclimation periods indicate that gluconeogenic pathway get activated to cope with the increasing metabolic demand at high temperatures. A consistent increase of transmainases might have provided the substrate for gluconeognesis [14,15]. However, chlorine induced suppression of this pathway is evident with increasing acclimation periods (at the end of 28 days) indicates that gluconeogenic pathway is affected in *C.carpio* fingerlings.

### 4.3. Effect on glycolytic pathway

Pyruvate kinase allows the free phosphates to react with ADP, to create new ATP molecules, which helps the unstable little sugar fragments to rearrange into stable pyruvate molecules, and subsequently leaves for aerobic or anaerobic pathway as per the requirement of the cell. FDPase in the liver, an example of feed forward stimulation, activates PK. In the present investigation, an increase in PK with increasing acclimation temperatures after 14 days indicates the high requirement of ATP in the cell for combating stress due to increasing temperatures. A consistent increase in FDPase confirms our above hypothesis. LDH activity showed a significant increase at higher temperature (36 °C). It may be due to the higher production of lactate, which indicates oxygen limited condition in the cell. However, LDH activity was inhibited due to free chlorine in rearing waters, which could be correlated with the absence of substrates (as transaminases and gluconeogenic pathway got affected). LDH activity appears stabilized after 28 days as compared to that of 14 days acclimation trial.

MDH, an enzyme of TCA cycle, was increased at higher acclimation temperature in order to use the product (oxaloacetate) due to the higher activity of AST for production of more energy (ATP), which may be utilized for other physiological activities. In the present study, there was a significant increase in MDH activity in fishes acclimated at higher temperatures, which strengthens the above hypothesis. Glucose might have therefore mobilized through non-carbohydrate source; mainly by protein, as transaminase activities were more at higher temperatures. Results also strengthen the fact that higher acclimation temperature induces amino acid mobilization (alanine and aspartate) in *C. carpio.* However, under the influence of chlorine, MDH activity appeared inhibited. Similar results were recorded when *Channa punctatus* were exposed to sub lethal levels of endosulfan [43].

### 4.4. Effect on pentose phosphate pathway

G6PDH catalyzes glucose-6-phosphate to 6-phosphogluconolactone using $NADP^+$ as a coenzyme and releases NADPH. This enzyme activity may increase when the requirement of NADPH increases inorder to synthesize fatty acid, cholesterol steroids and sphingolipids (lipid anabolism). NADPH produced also required by NADPH oxidase in producing superoxide anions for destroying phagocytosized material. So, the enzyme activity may increase with increased phagocytosis [40]. In the present study G6PDH activity was decreased in *C. carpio* at higher acclimation temperatures, which supports the findings of [44] in *Brook charr* after subjected to high stocking density. Similarly, increased concentration of cortisol inhibits the activity of G6PDH in fishes [45]. A comparison of acclimation period indicates and initial increase in control and chlorine treated groups after 14 days acclimation and subsequent decrease in G6PDH after 28 days, which might be an indirect indication of low levels of phagocytosis.

### 4.5. Effect on phosphate metabolism

ALP, a zinc-containing metallo-enzyme, which play an important role in metabolism of phosphorus in the body. In the present investigation, ALP activity in all the organs tested (intestine, liver, and muscle) increased significantly with increasing acclimation temperatures, which may be due to hydrolysis of high-energy phosphate bonds to liberate phosphate ions to combat stressful condition or higher metabolic rate. However, the presence of chlorine inhibited ALP activity in all the organs tested, which might be an indication of role of chlorine in inhibiting protein synthesis. Similarly, a dose dependent inhibition of ALP was reported in liver of *Zebra danios* exposed to malathion [46]. A consistent inhibition of ALP activity in liver, kidney and muscle was reported when *Channa gachua* was exposed to endosulfan, an organochlorine pesticide [47].

### 4.6. Effect on neurotransmission

AchE is one of the most widely used enzymes as a biomarker for environmental pollution. It is well known that

some pesticides (organophosphates and carbamates) inhibit AchE, which increases the acetylcholine and stimulates the medullary cells to release catecholamine [48] and also increases the aberrant behaviour [49,50]. Increased catecholamine may affect the activity of enzymes involved in glycogen synthesis and glycogenolysis. Thus, temperature has an effect on physiological parameters as well as neurotransmission. In the present study, significant inhibition of AchE activity was observed in brain tissue of *C. carpio* at higher acclimation temperatures and is augmented in chlorine treated groups, which strengthen the above hypothesis.

### *4.7. Effect on membrane transport in the cell*

ATPase is a membrane bound enzyme and is responsible for the transport of ions through the membrane and regulates $Na^+/K^+$ gradient along the cell membrane [18]. It helps in the carrier-mediated active transport of $Na^+$ and $K^+$ respectively outward and inward across the membrane. ATPase hydrolyzes the high-energy phosphate (ATP) and utilizes that energy to maintain ionic gradient across the plasma membrane [40]. The activity of ATPase might have got inhibited due to unavailability of substrate ATP. In the present investigation ATPase activity increased significantly with increasing acclimation temperatures, which indicates that there was no depletion of ATP between temperatures ranges 26–36 °C. Thus it regulates the energy balance in fishes [51], which is evident in present investigation. Strange et al., 1978, made similar observations. Presence of free chlorine ($0.1\,mg\,L^{-1}$) increased ATPase activity after 14 days acclimation, however stabilized after 28 days acclimation. Similar pattern of initial increase (42%) and subsequent decrease in ATPase activity was reported when *Channa punctatus* was exposed to sub-lethal doses of endosulfan [43].

## 5. Conclusion

Overall results indicate that *C. carpio* demonstrates metabolic readjustments with increasing temperatures, in order to cope with energy demand of the cell. However, chlorine exposure at higher temperatures affected protein metabolism, gluconeogenic and subsequently glycolytic pathway, leading to an energy-limited condition. In addition, alteration of membrane transport and neurotransmission can be considered as a prelude to cellular damage. Our investigation presents a vivid picture on the organ specific metabolic profiles of *C. carpio* fingerlings on exposure to chlorine and temperature. To our knowledge, there are no parallel reports on the influence of chlorine and high temperatures on metabolic profiles in *C. carpio.* However, the exact mechanism of action of chlorine on specific metabolic pathways remains elusive. Therefore, our *prima-facie* evidence on the impact of chlorine on temperature induced stress on fish model may invite attention of future researchers to investigate the mechanism of action of chlorine at various temperatures on aquatic organisms.

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