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Temperature induced biological alterations in the major carp, Rohu (*Labeo rohita*): Assessing potential effects of climate change on aquaculture production

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ABSTRACT

Temperature plays a profound role in organismal growth, immunity, survival, and the entire biological systems of any aquatic species. Suboptimal temperature changes modify different biological traits of fish that adversely affect their life cycle and aquaculture productivity. The freshwater Indian major carp, Rohu (*Labeo rohita*), is one of the most important aquaculture species across the Indian Sub-continent, with the optimum temperature range of 28 °C to 32 °C. This study tested the effects of five different temperature levels (28 °C, 30 °C, 32 °C, 33 °C and 34 °C) on selected physiological and biochemical traits, and also on the expression of five targeted genes of *L. rohita*. Experimental temperatures significantly affected growth performance (mean body weight) ($p < 0.05$), with the highest growth and survival observed at 30 °C. The lowest level of growth and survival were obtained at 34 °C, possibly due to thermal stress, despite the highest O_2 consumption rate and total blood cell counts found at this temperature. A significantly lower growth ($P < 0.05$) was observed at 28 °C (control) compared to 30 °C. The five target genes including growth hormone (GH) and insulin like growth factor I (IGF-I) as growth regulatory genes, glycerol-3-phosphatase (G-3-P) and Gherlin (as metabolic genes), and heat shock protein 70 (HSP70 as thermal stress response gene), showed variable expression levels with varying temperatures. The growth genes showed the highest expression at 28 °C and 30 °C, whereas the stress response gene showed the highest expression at 34 °C. Results of this study indicate that Rohu can perform well up to 32 °C without any adverse effects, with optimal production performance occurring between 28 °C and 30 °C. This further implies that appropriate measures must be in place to maintain optimum temperature in the aquaculture farms such as physically manipulating the production system to control temperature or through development of temperate tolerant strains in order to avoid potential negative consequences of climate change.

1. Introduction

Climate change has been a global concern since the mid-nineteenth century, resulting in extreme weather events and abrupt environmental changes that are increasingly more likely to occur in future (Islam et al., 2020). Extreme alterations in climatic factors such as temperature and precipitation rates have been accelerated by anthropogenic activities, causing rapid fluctuations in temperature and salinity levels in water bodies (Shahjahan et al., 2021; Yilmaz et al., 2021). This, in turn, creates hydrological stressors on aquatic organisms, particularly those living in small and shallow water bodies (i.e. aquaculture species) (Sabbir et al., 2010; Cox et al., 2018; Armobin et al., 2023). Thus, climatic stressors significantly impact aquatic ecosystems, biodiversity, and aquaculture, as well as the associated human communities (Islam et al., 2011; Rahi et al., 2013; De et al., 2019). Particularly impacted will be the freshwater aquaculture sector because most freshwater species require high-quality water and stable environmental conditions (Phuc et al., 2017; Ninawe et al., 2018; Rahman et al., 2022). Deviation from the optimal water temperature imposes stress stimuli, reducing O_2 levels, increasing susceptibility to disease, and altering metabolic and

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reproductive performance (Serezli et al., 2017; Georges and Holleley, 2018; Uppanunchai et al., 2018; Rahi et al., 2021a). Therefore, a significant amount of research is concerned with modulating water temperature in order to predict the potential future consequences to aquaculture.

Temperature changes affect the entire biological organization of aquatic organisms. This may include: physiological traits (growth, feed intake, metabolism, reproduction, behavior); biochemical parameters (changes in blood biochemical composition, glucose and hormonal imbalance, blood cell lysis); and genetic aspects (increased expression of stress response genes and consequent decrease in the expression of other candidate production-associated genes), that make fish extremely vulnerable to disease and mortality (Islam et al., 2015; Rahi, 2017; Shahjahan et al., 2018; Dawood et al., 2020; Rahi et al., 2021b). It has been estimated that a 1 °C change in water temperature can alter a fish's metabolic rate by 10% (Kato et al., 2011; Darnell et al., 2013; Chengsong et al., 2015; Yamamoto et al., 2015). Growth performance of different fish species has been found to decrease at low water temperatures, while growth improves with rising temperatures within the tolerance limit (Huang and Chou, 2015; Rahi et al., 2021b). Fish endure mass mortality during extreme winter and summer seasons by modifying their energetic costs for internal biological regulation or thermoregulation (Sadoul and Geffroy, 2019; Shen et al., 2018; Islam et al., 2020). Thus, water temperature beyond organismal optimal tolerance limits leads to deleterious effects on specific organisms and biodiversity (Wang et al., 2018; Rahi et al., 2021c).

The ability of aquatic species to respond rapidly to temperature change determines the persistence of the species in the ecosystem as well as its future aquaculture potential (Ali et al., 2009; Maulvault et al., 2017). Important physiological and biochemical indicators for detecting the stress level and stress response mechanisms include changes in the concentration of several critical blood plasma parameters (e.g., glucose, cortisol, total protein, triglycerides, lactate and compositions of different ions) (Almeida et al., 2015; Shahjahan et al., 2018; Asha-f-Ud-Doulah et al., 2019). Moreover, molecular markers (e.g., mRNA expression levels of genes associated with stress response, growth, metabolism and immunity) serve as sensitive indicators for understanding the severity of imposed stress as well as the spectrum of stress mitigation pathways (Eissa et al., 2017; Hossain et al., 2018; Rahi et al., 2022).

Globally, inland freshwater aquaculture is extremely vulnerable to increasing water temperature due to climate change (Rahi and Shah, 2012a; Shahjahan et al., 2018; Afroz et al., 2021). The aquaculture sector represents a vital source for the economy of many countries; for example, this sector acts as the second largest export earning source for Bangladesh (Sabbir et al., 2017; Rahi et al., 2022). The Indian major carp, Rohu (*Labeo rohita*), is one of the most important freshwater aquaculture species and a popular fish food item across the entire Indian subcontinent, contributing to 10.54% of Bangladesh's total fish production with an estimated local market value of ≈US$ 925 million (FSY, 2021; Rahi et al., 2012a; Islam et al., 2015). In addition, Bangladesh earns US$ 100–150 million annually by exporting the fry/seed of this species to other Southeast Asian countries (Shah et al., 2011; Ali et al., 2015; DoF, 2021). Besides, this species plays a vital role in the livelihood of ~5 million people across Bangladesh (Ali et al., 2008; Afroz et al., 2021). Bangladesh is one of the most vulnerable countries that is likely to be severely affected by climate change events because of its unique geographical positioning between the fury of both the Bay of Bengal (to the south) and the Himalayas (to the north) (Chen and Hu, 2018). Therefore, the increasing water temperature will severely affect the inland aquaculture sector across Bangladesh, including the farming of Rohu.

Rohu is an obligate freshwater species (its entire life cycle is confined to freshwater environments); is a mid-layer water column inhabitant. Growth performance in culture suggests that they prefer relatively stable water temperatures ranging from 28 to 30 °C (Rahi and Shah, 2012b; Roychowdhury et al., 2019). Thus, more extensive changes (increase) in water temperature can pose thermal stress at different levels of magnitude depending on the intensity of temperature rise. During the summer season, water temperature in aquaculture ponds can reach 36 °C, which is almost 6 °C higher than the optimal range for Rohu, making it imperative to examine the thermal stress compensatory mechanisms. Previous investigations observed the effects of higher temperatures (33 °C, 35 °C and 36 °C) on different biological traits of Rohu (Islam et al., 2020; Shahjahan et al., 2021). Results of earlier studies showed adverse effects on growth, survivability and blood parameters at 35 °C and 36 °C. It is thus, urgently warranted to detect the maximum thermal tolerance limit for Rohu that will help to optimize aquaculture production in light of predicted temperature increases associated with climate change. Therefore, the objectives of the present study were to investigate the physiological (different growth parameters, O_2 consumption and survival), biochemical (blood cell counts, blood glucose and cortisol hormone concentrations), and genetic (expression levels of five genes associated with growth, metabolism, immunity and thermal stress tolerance) indices of Rohu at five different temperature levels (28 °C, 30 °C, 32 °C, 33 °C, and 34 °C) to determine the levels of stress caused by specific temperature and stress response mechanisms.

2. Materials and methods

2.1. Experimental Rohu collection

Juveniles (fingerlings) of Rohu (mean body weight ≈ 0.5 g for each) were collected from a government hatchery (Fish Seed Multiplication Farm), Khulna, Bangladesh. In total, 375 Rohu fish samples (from the same cohort) were collected from the nursery rearing pond of the hatchery and then transported to of the Fisheries and Marine Resource Technology (FMRT) Discipline wet laboratory, Khulna University, in a plastic bag filled with oxygen.

2.2. Tank preparation and acclimatization

Fifteen glass tanks (30 L each) were filled up to 25 L using water from the same nursery pond and maintained with continuous aeration for three days. In each experimental tank, 25 Rohu fingerlings were randomly allocated (75 fish per temperature). The fish were fed with a commercial nursery feed (CP Co. Ltd; 33% crude protein content) at the rate of 10% of total biomass. Feed was applied twice daily at 7.00 am in the morning and 7.00 pm at evening. Bottom debris (uneaten feed particles and excreta) were siphoned out daily from the tank to maintain optimum water quality in the tanks. Elongated (threat like) structures were considered as excreta and separated from uneaten feed particles. Uneaten feed particles were oven dried (at 60°C) which was considered as 'dry remaining diet recovered' to precisely calculate the feed intake (FI). An automated thermostat (RSS-499, China) was set in each tank to maintain 28 °C (as the control temperature) for acclimating the experimental fish to the tank environment for 10 days before the experiment commenced.

2.3. Temperature stress experiment

Five different temperature treatments (28 °C, 30 °C, 32 °C, 33 °C and 34 °C) in triplicate tanks were used for this study by using automated thermostats. Following the 10 day acclimation period, the temperature was raised by 2 °C per day (temperature was increased by 1 °C at every 12 h interval to impose minimum thermal stress) to achieve the target temperature levels (four different treatments). To achieve the target temperature treatments, it took 24 h for 30 °C, 48 h for 32 °C, 60 h for 33 °C and 72 h for 34 °C. For 34 °C, temperature was raised 2 days before 30 °C and 1 day before 32 °C to achieve the target temperatures simultaneously. After reaching each temperature threshold, the experiment was carried out for 60 days.

2.4. Evaluation of survival rate and growth performance

Experimental Rohu fingerlings were sampled every fortnight (30 individuals at each sampling time; 10 individuals from each replicate tank) to measure body weight. Fishes were released in the experimental tanks after growth measurement. For the biochemical and gene expression study, three fishes were dissected at each sampling time that sacrificed 27 fish in total. Survival rates were estimated by counting the number of individuals alive at the end of the 60 day experiment. The following equations were used to measure different growth parameters:

$$\text{DWG } (\%) = \{(BW_f - BW_i)/(BW_i \times t)\} \times 100$$

$$\text{SGR } (\%) = \{(\ln BW_f - \ln BW_i)/t\} \times 100$$

$$\text{FCR} = \text{feed intake (g)/weight gain (g)}$$

$$\text{FI (g fish}^{-1}\text{ day}^{-1}) = \text{(dry diet given – dry remaining diet recovered) / number of fish}$$

$$\text{PER} = \{\text{live weight gain (g)/ dry protein intake (g)}\} \times 100$$

$$\text{TGC} = [\{\sqrt[3]{(BW_f)} - \sqrt[3]{(BW_i)}\}/ \text{(temperature} \times \text{number of days)}] \times 1000$$

Here, DWG = daily weight gain, BW_f = final body weight, BW_i = initial body weight, t = total experimental time (60 days), SGR = specific growth rate, FCR = feed conversion ratio, PER=Protein Efficiency Ratio, FI=Feed Intake and TGC= Thermal Growth Coefficient.

2.5. Rates of oxygen consumption

The experimental fish samples were collected at nine different time intervals to measure temperature dependent changes in the rate of O_2 consumption. The sampling times were Day 1 (immediately after achieving target temperatures), Day 2, Day 3, Day 4, Day 5, Day 10, Day 20, Day 30 and Day 60. Three replicated fish (one from each tank) were used for measuring O_2 consumption rates according to Rosas et al. (2001) and Rahi et al. (2020). The same temperature (as maintained in experimental tanks) was maintained in respirometric chamber using the thermostat to precisely determine temperature specific O_2 consumption changes. A 250 mL flow respirometric chamber (Q-Box Aqua Respiratory System, Qubit, Canada) was used to measure temperature-specific O_2 consumption rates. Experimental fishes were placed in the respirometric chambers three hours before measuring O_2 consumption rates to avoid effects of handling/transferring stress. Only a single fish was placed in each chamber to precisely measure the O_2 consumption rate. The water flow rate (FR) was 1.5 Lh^{-1} in each chamber. O_2 consumption rates were measured by deducting the amount of O_2 during exit (O_2 ex) from O_2 at entry (O_2 en) using the following equation:

$$O_2 \text{ consumption} = [O_2 \text{ en} - O_2 \text{ ex}] \text{ x FR}$$

After measuring the O_2 consumption, experimental fishes were weighed instantly to quantify the O_2 consumption rates (mg O_2 $hr^{-1}g^{-1}$). Three replicated fish were used each time from each temperature to measure O_2 consumption rates.

2.6. Total blood cell counts

Three replicated fish samples were collected from each temperature (one from each tank) for blood collection. Blood samples (50 μL) were collected from each fish by micro-injection and were immediately transferred to Eppendorf tubes containing equal volumes of anticoagulant (20 mM EDTA). The anticoagulated blood samples (100 μL) were fixed for 30 min using an equal volume of neutral buffered formalin (10%) to measure the total blood cell count (TBC). The fixed samples were serially diluted for 2, 4, 8, 16, and 32 times using ice-cold phosphate-buffered saline (PBS, 20 mM, pH 7.2) (Rajendiran et al., 2016; Dawood et al., 2020). The total number of blood cells was then counted at 100 ×magnification using a hemocytometer (Boeco, Hamburg, Germany) placed under a microscope (SOLARIS-TLED, Rome, Italy).

2.7. Determination of blood glucose and cortisol concentrations

Blood samples were collected from the experimental fishes (three replicated fish per temperature) using heparinized syringes according to Pravda and Svobodová (2003) and immediately transferred to the tubes containing heparin (50 IU/mL of blood) (Zentiva, Czech Republic) to avoid blood clotting. For assaying glucose levels, 100 μL of blood was collected. Blood plasma was separated by centrifuging the samples at 800 g for 10 min (at 4 °C); the separated plasma samples were then stored at −80 °C for subsequent analysis. The glucose levels of Rohu blood plasma samples were determined using a commercial kit (Glu L 1000, PLIVA-Lachema, Czech Republic). Another 200 μL of blood was drawn from each fish (same fish that were used for blood cell counts and glucose determination) to determine plasma cortisol levels. Blood samples were centrifuged at 16,000 g for 2 min at 4 °C to separate plasma from each sample. The plasma collected from each sample (80 μL) was then frozen in liquid nitrogen and preserved at −80 °C until use. Finally, plasma cortisol levels were estimated by monoclonal antibody enzyme-linked immunosorbent assay (ELISA) quantification kit ((Enzo Life Sciences, Farmingdale, NY, USA) according to Fuchs et al. (2015), Bögner et al. (2018) and Islam et al. (2020).

2.8. Gene expression analysis

Due to the small size of experimental fish, whole individuals of Rohu were used to investigate the expression pattern of different genes. As previously identified, samples were collected at nine different times, where Day 1 indicates sampling immediately after exposure to different temperature levels. Three replicate individuals were collected at each sampling time from each temperature treatment. Total RNA was extracted using the TRIzol/chloroform extraction method, followed by RNA purification using a commercial RNA extraction kit (Qiagen, Germany) according to the manufacturer's protocol. RNA purity and quantity were evaluated using 2% agarose gel electrophoresis and a NanoDrop 2000 Spectrophotometer (Thermo Scientific, USA). High-quality RNA samples were preserved at −80 ℃ for subsequent use. RNA samples (1 μg RNA for each sample) were used for complementary DNA (cDNA) synthesis by using a SensiFAST cDNA synthesis kit (Bioline, UK) according to the manufacturer's protocol and preserved at −20℃ for further analysis.

Five different genes with distinct functional roles were used for the RT-qPCR-based gene expression study. These included growth regulation genes (growth hormone (GH) and insulin like growth factor I (IGF-I)), metabolic performance genes (glycerol-3-phosphatase (G-3-P) and Gherlin), and a thermal stress response gene (heat shock protein 70 (HSP70)). Elongation factor 1 alpha (EF 1α) was used as the reference gene (Sahu et al., 2015). Specific details of the genes are given in Table 1. The target genes were chosen based on their functional roles elucidated in previous research (Sahu et al., 2015; Islam et al., 2020). Sequences of the target genes were obtained from an earlier study (Sahu et al., 2015) and loaded into Primer3 software (Untergasser et al., 2012) to design gene-specific primers (Table 1). RT-qPCR reactions were performed in 20 μL mixtures including ultra-pure water (3 μL), cDNA as template (5 μL), forward primer (1 μL), reverse primer (1 μL), and 10 μL of 2x SensiFAST SYBR No-ROX Mix (Bioline, UK). RT-qPCR reactions were performed in a real-time PCR system (Bio-Rad, California, USA) using three technical replicates for each sample. Following the reactions, a standard melt-curve analysis was performed to confirm the amplification of a single qPCR product. Data obtained from the RT-qPCR were analyzed (relative expression level of each gene) using the ΔΔCt method (Pfaffi, 2001; Kokou et al., 2019) according to the following equation:

$$\text{Relative Gene Expression (R)} = 2^{-[\Delta Ct \text{ Target Gene} - \Delta Ct \text{ Reference Gene}]}$$

Table 1
Specific details of the primers for the selected candidate genes (Sahu et al., 2015; Shahjahan et al., 2021) used in this study. F = Forward, R = Reverse.

Candidate Gene	Primer Sequence (5′ – 3′)	Product length (bp)	Annealing temperature	Amplificancy efficiency
Insulin like growth factor I (IGF-I)	**F:** CCCGGGGTCAAAATGCAGCT **R:** GGGGTAACTCAGGCCACGGA	120	57ºC	0.97
Growth Hormone (GH)	**F:** AGAAGCTCTTGCAGCCCTCG **R:** CCACAGAACGTGTTGCGGGA	118	59ºC	0.99
Glycerol-3-phosphate (G-3-P)	**F:** CGTCCTGTTCACTGCACCCAG **R:** ATGCCACAGCAGACGTCGCT	112	60ºC	0.98
Gherlin	**F:** TGCAGGTCTCTGTGGTGGTG **R:** ACAGCTGGATGCTGGGCAGT	130	61ºC	0.98
Heat Shock Protein 70 (HSP 70)	**F:** CTACTCGGACAATCAGCC **R:** GGAATGCCAATCAACTCA	105	54ºC	0.99
Elongation factor 1 alpha (EF 1α)	**F:** TTCGAGCAGGAGATGGGCACTG **R:** GCATCCTGTCAGCAATGCCA	114	60ºC	0.98

2.9. Statistical Analysis

All the data were checked for normality and homogeneity of variance using Kolmogorov-Smirnov and Levene's tests, respectively using the SPSS (Version 23) software. Data generated for different parameters (growth, O_2 consumption, blood cell counts, glucose and cortisol levels, and gene expression) were used for One- and Two-Way analysis of Variance (ANOVA). Temperature and sampling times were considered as independent variables while the biological parameters were considered as the dependent variables for Two-Way ANOVA. One-Way ANOVA was performed to test the statistical significance for the biological parameters across different sampling times. The R package (version 3.5.1) was used for various types of correlation plotting between different parameters. Significant differences ($P < 0.05$) between the means of different parameters were evaluated using the Tukey HSD test. Results in tables and graphs are presented as mean ± standard error (SE).

3. Results

3.1. Temperature-induced changes in growth performance and survival

Different temperature treatments significantly affected the growth performance and survival ($F_{(4, 150)} = 96.4$, $P = 0.00$) of experimental Rohu individuals across sampling times (Table 2 and Fig. 1). Significantly higher growth performance was observed at 28 °C – 32 °C temperature range compared to 33 °C and 34 °C temperatures throughout the experiment. The highest level of growth was observed at 30 °C (significantly greater than all other temperatures, except for 28 °C).

Table 2
Effect of different temperature levels on growth parameters (mean ± SD) of Rohu (*Labeo rohita*). Different superscripts indicate significant differences ($P < 0.05$).

Growth Parameters	28 °C	30 °C	32 °C	33 °C	34 °C
Initial Weight (BW_i) (g)	0.4^a ± 0.08	0.4^a ± 0.07	0.4^a ± 0.09	0.4^a ± 0.08	0.4^a ± 0.07
Final Weight (BW_f) (g)	2.39^a ± 0.6	2.61^b ± 0.9	2.31^a ± 0.8	1.91^c ± 0.7	1.71^d ± 0.7
Daily Weight Gain (DWG) (%)	5.29^a ± 1.3	6.04^b ± 1.4	5.17^a ± 1.2	3.70^c ± 1.1	3.63^c ± 1.1
Specific Growth Rate (SGR) (%)	2.38^a ± 0.6	2.56^b ± 0.7	2.35^a ± 0.6	2.03^c ± 0.6	1.93^c ± 0.5
Feed Intake (FI) (g g^{-1} day^{-1})	0.153^a	0.156^{ab}	0.159^b	0.161^b	0.162^b
Feed Conversion Ratio (FCR)	1.41^a ± 0.3	1.22^b ± 0.2	1.36^a ± 0.2	1.94^c ± 0.4	2.01^c ± 0.4
Protein Efficiency Ratio (PER)	1.88^a ± 0.5	2.09^b ± 0.7	2.11^b ± 0.8	1.55^c ± 0.4	1.02^d ± 0.3
Thermal Growth Coefficient (TGC)	1.73^a ± 0.3	1.96^b ± 0.4	1.99^b ± 0.4	1.43^c ± 0.2	1.39^c ± 0.2
Survival Rate (%)	100^a	91^b	83^c	71^d	62^e

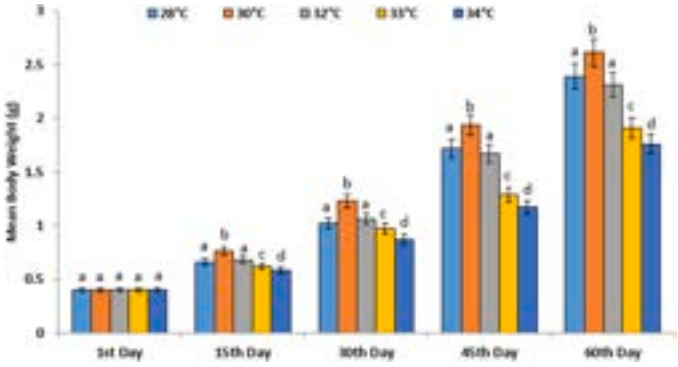

Fig. 1. : Mean body weight (± S.E.) of Rohu (Labeo rohita) at 15 days intervals with increasing temperature. Different letters above the error bar indicate significant differences ($P < 0.05$).

Other growth-related parameters, such as daily weight gain (DWG), specific growth rate (SGR), feed intake (FI), feed conversion ratio (FCR), protein efficiency ratio (PER), thermal growth coefficient (TGC) and survival rate (Table 2) exhibited similar trends as observed for the growth performance (Fig. 1).

3.2. Changes in the rate of O_2 consumption

Experimental temperature regimes significantly affected the O_2 consumption rates of Rohu ($F_{(4, 180)} = 102.5$, $P = 0.00$) (Fig. 2). At the beginning of this trial, a significant difference was observed only between 28 °C and 34 °C (Fig. 2). From Day 2 through to the end of the experiment (60th day), all other temperature treatments (30 °C, 32 °C, 33 °C, and 34 °C) showed significantly higher O_2 consumption rates ($P < 0.05$) than the control (28 °C). The O_2 consumption rate among the temperature treatments increased significantly ($P < 0.05$) until the third day (the peak level), then declined until the fourth day, and remained stable for the remainder of the experiment. A significant ($P < 0.05$) and positive correlation ($R^2 = 0.13$) was observed between growth and O_2 consumption (Fig. 11).

3.3. Blood Cell Counts

Significant variations in the blood cell counts were observed ($F_{(4, 180)} = 87.3.2$, $P = 0.01$) among the Rohu fingerlings maintained under different experimental temperatures across different sampling times (Fig. 3). A significantly higher number of blood cells were obtained at 28 °C compared to the other temperatures ($P < 0.05$) from the beginning to the second day. However, no significant differences were observed among the temperatures on the third day. No significant differences were observed between 30 °C and 28 °C (control) throughout the experiment. In comparison, the remaining three temperatures

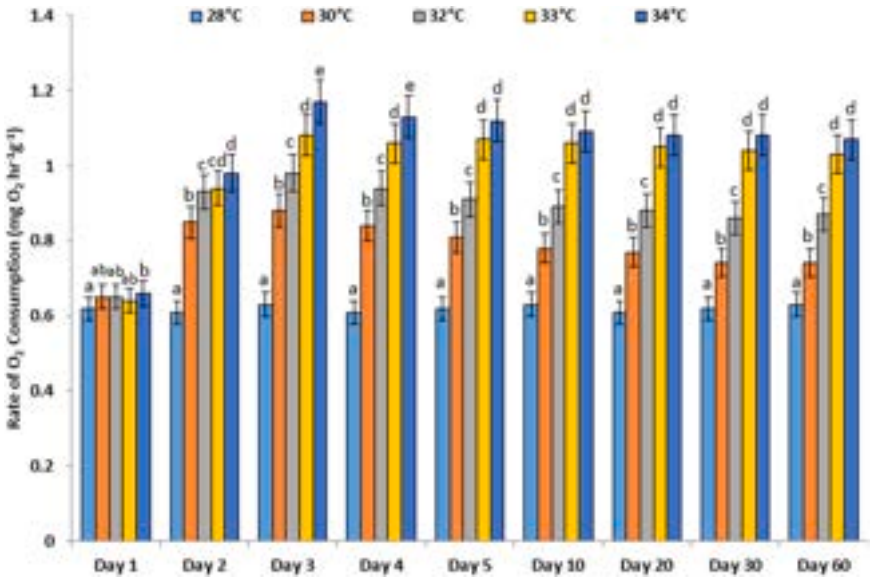

Fig. 2. : Temperature induced changes (Mean ± S.E.) in the rate of O_2 consumption in Rohu (Labeo rohita) across sampling times. Different letters above the error bars indicate significant differences ($P < 0.05$).

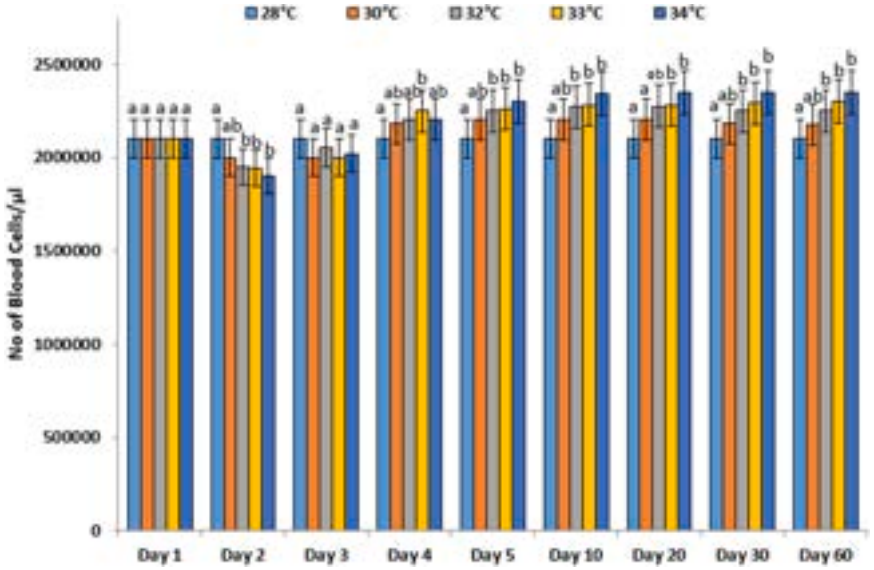

Fig. 3. : Changes in blood cell counts with increasing temperature in Rohu (Labeo rohita) juveniles across the sampling times.

(32 °C, 33 °C, and 34 °C) showed significantly higher blood cell counts ($P < 0.05$) over the control (28 °C) from the fourth day to the end. No significant differences were observed among 32 °C, 33 °C, and 34 °C temperature treatments from the beginning to the end of this experiment.

3.4. Changes in blood cortisol levels

The stress hormone (cortisol) levels in the blood of Rohu were significantly affected by temperature treatments ($F_{(4, 180)} = 91.7.2$, $P = 0.00$) in the current study (Fig. 4). At the control temperature, significantly lower but steady levels of cortisol were found ($P < 0.05$) throughout the experiment. The experimental fish at 30 °C, 32 °C, 33 °C, and 34 °C had significantly higher cortisol levels ($P < 0.05$) than the control (28 °C) throughout the trial. The cortisol levels of temperature treated (30 °C - 34 °C) Rohu increased significantly until the fifth day and then remained stable from 10th day to the end of the trial (Fig. 4). No significant differences were detected between 33 °C and 34 °C until the fourth day, after which significant differences ($P < 0.05$) were observed for the remainder of the experiment.

3.5. Changes in blood glucose levels

Similar to changes in cortisol levels, blood glucose levels were also significantly affected by temperature treatments and sampling times ($F_{(4, 180)} = 92.6.2$, $P = 0.00$). Compared to the individuals maintained at 28 °C temperature (control), fish exposed to 33 °C and 34 °C treatments exhibited almost double the levels of glucose in the blood. In contrast, Rohu exposed to temperatures of 30 °C and 32 °C exhibited glucose levels almost 1.5 times higher (Fig. 5). Glucose levels increased significantly ($P < 0.05$) until the fifth day (peak level), then declined until the 30th day, and then stabilized. From the second day until the end of the experiment, glucose levels were significantly higher ($P < 0.05$) at 33 °C and 34 °C compared to the other temperature levels, although no

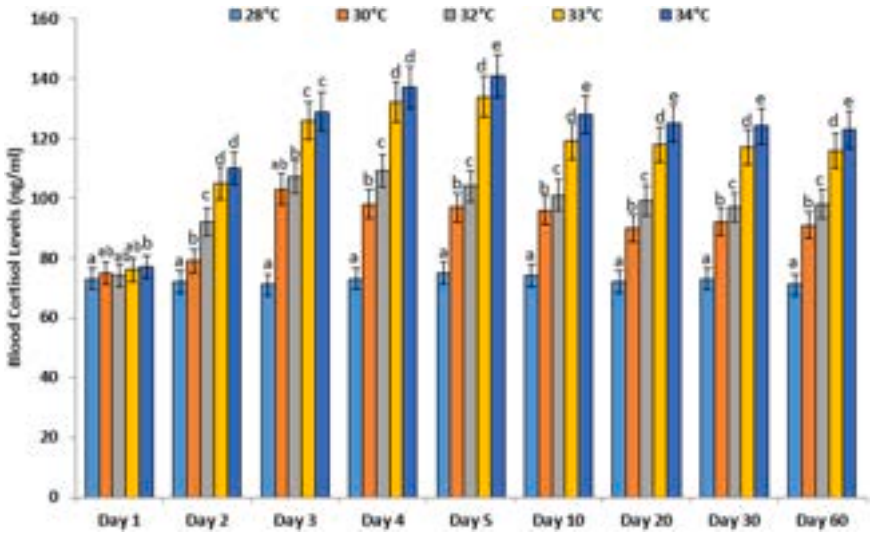

Fig. 4. : Changes in blood cortisol levels with increasing temperature in experimental Rohu (Labeo rohita) across the sampling times.

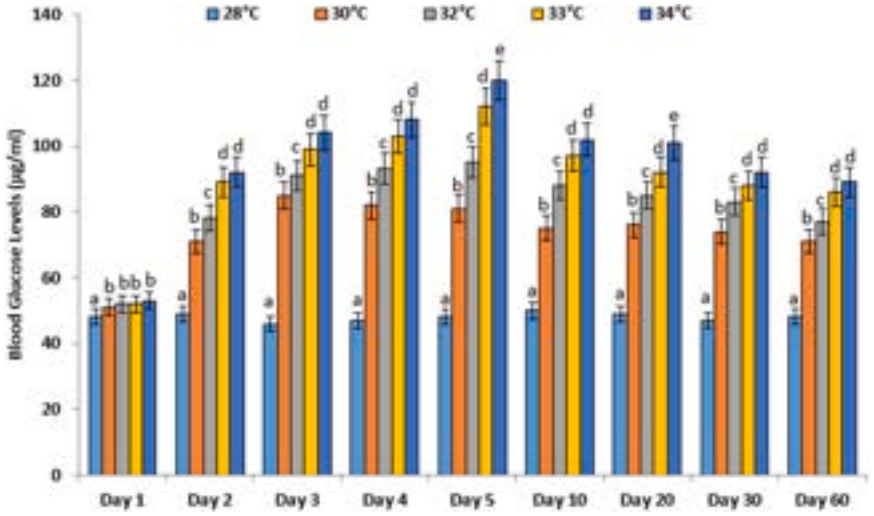

Fig. 5. : Changes in the blood glucose level with increasing water temperature in experimental Rohu (Labeo rohita) fishes across the sampling times.

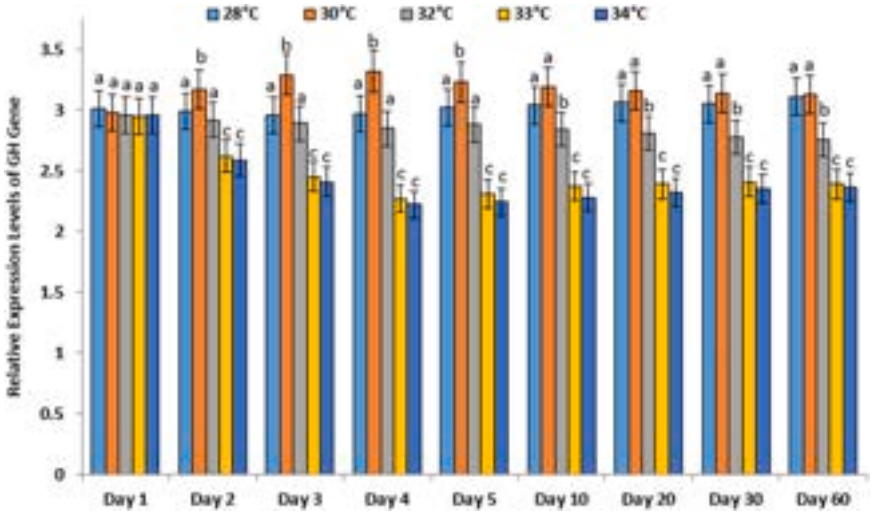

Fig. 6. : Changes in relative expression levels of growth hormone (GH) gene (relative to the reference gene, EF 1α) with increasing temperature in Rohu (Labeo rohita) juveniles across the sampling times.

significant differences were observed between these two temperature treatments throughout (except for the 5th and 20th day) (Fig. 5). Significant differences ($P < 0.05$) were also observed between 30 °C and 32 °C from second day until the end. Fig. 12 shows statistically significant ($P < 0.05$) positive correlation for growth with glucose and cortisol levels ($R^2 = 0.72$ for cortisol and $R^2 = 0.734$ for glucose) throughout the experiment.

3.6. Relative expression levels of target candidate genes

Temperature treatments significantly altered the expression levels of the five candidate genes tested in this study (Figs. 6 – 10). Significant effects were observed for the five genes with temperature treatments and sampling times ($F_{(4, 180)} = 67.4 – 79.8$, $P = 0.01 – 0.03$). The expression levels of both growth regulatory genes (GH and IGF-I) were significantly greater at 28 °C and 30 °C compared to the other three temperature treatments (Figs. 6 and 7). The maximum GH and IGF-I expression levels were observed at 30 °C, whereas the lowest levels were observed at 34 °C. Initially (1st day), there were no significant differences among the experimental temperatures for both the GH and IGF-I. At 30 °C, the GH gene exhibited significantly higher expression levels than the control until the fifth day, but IGF-I expression levels were significantly higher from the third to the 30th day. No significant differences were observed between 33 °C and 34 °C throughout the experiment for GH, while significant differences were observed ($P < 0.05$) for these two temperature treatments from the second day to the end of the trial for IGF-I.Fig. 11.

In contrast to the growth genes, the metabolic gene (G-3-P) showed a reverse pattern in its expression levels, in which the four higher temperature groups (30 °C, 32 °C, 33 °C and 34 °C) showed significantly higher ($P < 0.05$) expression over the control (28 °C) (Fig. 8). At the commencement of this trial (on the first day), no significant differences were found between the treatment and control groups; nevertheless, the treatment groups exhibited significantly greater expression ($P < 0.05$) than the control throughout the experiment. Rohu at 30 °C and 32 °C did not differ significantly ($P < 0.05$) throughout the trial with two exceptions (on the 2nd and 60th day) (Fig. 8). No significant differences were observed between 30 °C versus 32 °C and 33 °C versus 34 °C comparisons from the second day of this study until its completion (Fig. 8). From the second day to the end, experimental Rohu exposed to temperatures of 33 °C and 34 °C exhibited significantly higher G-3-P expression levels than those exposed to 30 °C and 32 °C..

Similar to the metabolic genes, temperature treatments had a significant impact on the expression levels of the thermal stress response gene (HSP70) of *L. rohita* fingerlings (Fig. 9). The HSP70 expression level of the control group (28 °C) was steady and significantly lower ($P < 0.05$) than that of the treatment groups (30 °C, 32 °C, 33 °C and 34 °C) from the beginning to the end of the experiment. Expression of HSP70 was found to reach its peak level rapidly (on the third day) but took longer time (from 20th day to the end) for the treatment groups to establish stability (Fig. 9). All treatment groups showed significant differences in expression levels for HSP70 from 2nd day to the end with a few exceptions (2nd day and 60th day).

The expression levels of the neuropeptide ghrelin showed remarkable differences with temperature treatments (Fig. 10). At the control temperature (28 °C), experimental Rohu showed significantly higher expression levels than the other four treatment groups from the second day to the end (the sole exception being that there was no significant difference between 28 °C and 30 °C from the 20th day until the end). Similar to the control temperature, 30 °C and 32 °C treatments showed significantly higher expression levels ($P < 0.05$) compared to 33 °C and 34 °C treatments (Fig. 10) throughout the experiment. No significant differences were observed between 30 °C and 32 °C until the 20th day, after which 30 °C showed significantly higher expression than 32 °C. Similarly, no significant differences were obtained between 33 °C and 34 °C treatments until the second day, after which expression at 33 °C significantly outperformed 34 °C. Fig. 13 represents significantly ($P < 0.05$) negative interactions ($R^2 = -0.688$ for GH, $R^2 = -0.713$ for IGF-I and $R^2 = -0.743$ for ghrelin) between the growth and expression of GH, IGF-I and ghrelin. In addition, significant ($P < 0.05$) and positive interactions of HSP70 with blood cortisol and glucose levels of Rohu ($R^2 = 0.34$ for cortisol and $R^2 = 0.46$ for glucose) were observed in Fig. 14.

4. Discussion

Various parameters evaluated in this experiment (O_2 consumption, glucose and cortisol levels, blood cell counts and gene expression) were found to be relatively stable at the control temperature (28 °C), indicating that Rohu (*L. rohita*) acclimated well to this temperature. Consequently, any deviation from this control temperature will reflect temperature specific alterations in the expression of different traits. Significant differences across experimental groups (Table 2 and Fig. 1) demonstrate that temperature stress adversely affects growth and survival rates. Stable O_2 consumption rates (Fig. 2) indicate no imposed

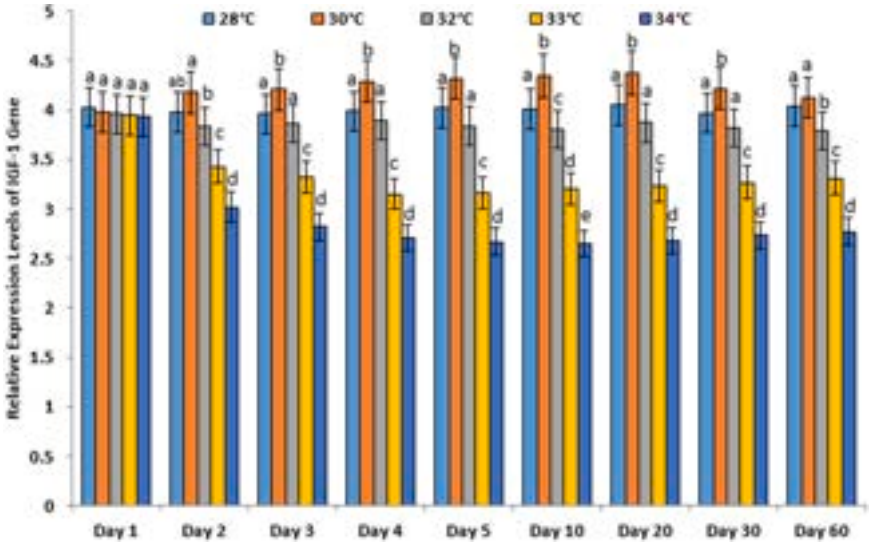

Fig. 7. Changes in relative expression levels of insulin like growth factor 1 (IGF-1) gene (relative to the expression of EF 1α) with increasing temperature in Rohu (Labeo rohita) juveniles across the sampling times.

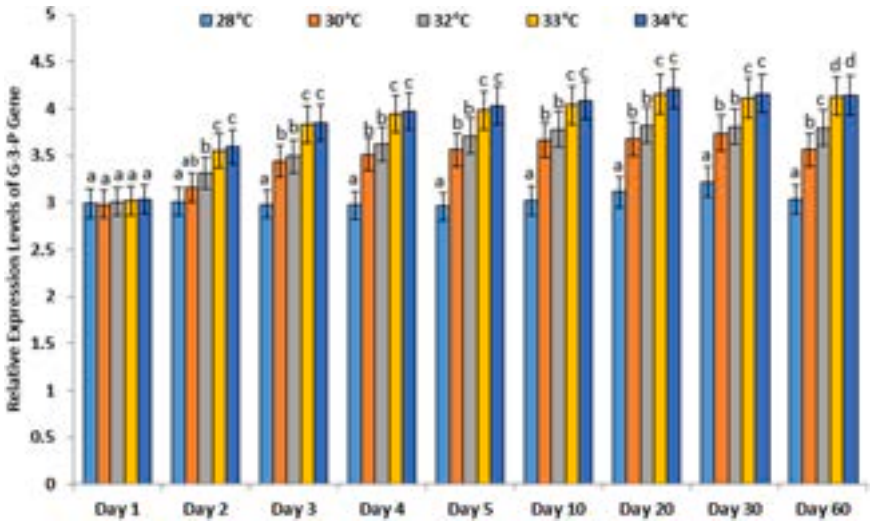

Fig. 8. Changes in relative expression levels of Glycerol-3-phosphate (G-3-P) gene (relative to EF 1α) with increasing temperature in Rohu (Labeo rohita) juveniles across the sampling times.

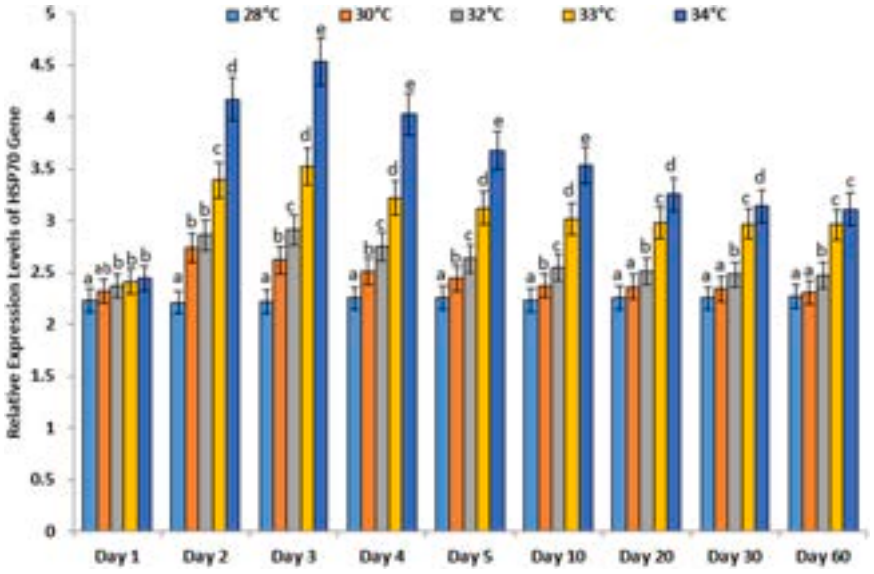

Fig. 9. Changes in relative expression levels of HSP70 (relative to EF 1α) with increasing temperature in Rohu (*Labeo rohita*) juveniles across the sampling times.

stress at 28 °C (control). Significant and positive correlation between growth and O_2 consumption (Fig. 11), implying that O_2 consumption plays a vital role in growth performance of Rohu.

In general, elevated O_2 consumption indicates increased metabolic processes that enhance growth (Rahi, 2017; Acquafredda and Munroe, 2020; Little et al., 2020; Rahi et al., 2021a). Under stressful conditions, fish also consume higher rates of O_2 to meet the additional demand for energy to counteract the adverse effects of any stressors (Rajendiran et al., 2016; Aziz et al., 2017; Rahi et al., 2020; Thawinwan et al., 2022). Even though Rohu individuals in the treatment groups (30 °C, 32 °C, 33 °C and 34 °C) consumed more O_2 than the control (Fig. 2), growth and survival rates were significantly lower at 32 °C, 33 °C and 34 °C (Table 2 and Fig. 1). These results suggest that higher temperature induced thermal stress on Rohu that drastically retarded growth and also represents stress mitigation strategies. Superior growth at 30 °C might indicate a maximum thermal tolerance limit at which Rohu could grow without experiencing detrimental consequences.

Blood cell counts reveal the degree of imposed stress as well as the immunity status (Hayashi et al., 2021; Seibel et al., 2021; Armobin et al., 2023). Initially, a fast drop in blood cell counts (Fig. 3) at higher temperatures (30 °C, 32 °C, 33 °C and 34 °C) indicated thermal stress induced blood cell lysis (Fig. 3). Eventually, an increasing trend in the number of blood cells indicated an acclimation strategy to elevated temperatures by the experimental fish. When an organism is under stress, O_2 requirement increases to compensate for the imposed stress (Chaudhry et al., 2021). Moreover, increasing water temperature reduces dissolved oxygen levels in water (Rahi et al., 2021a). In this regard, organisms tend to increase the number of blood cells to transport and hold more O_2 within the cells (Rahi et al., 2018; Volkoff and Ronnestad, 2020; Ashry et al., 2021). This presumably explains why the number of blood cells increased in Rohu fingerlings exposed to warmer temperatures. The greater number of blood cells of Rohu at higher temperatures is also indicative of the magnitude of thermal stress exerted on the experimental subjects; the highest stress level occurred at 34 °C.

The vertebrate stress hormone (cortisol) and blood glucose levels are crucial biochemical indicators for determining the severity of stress in fish (Martínez-Mota et al., 2007; Esmaeili, 2021; Rahi et al., 2022).

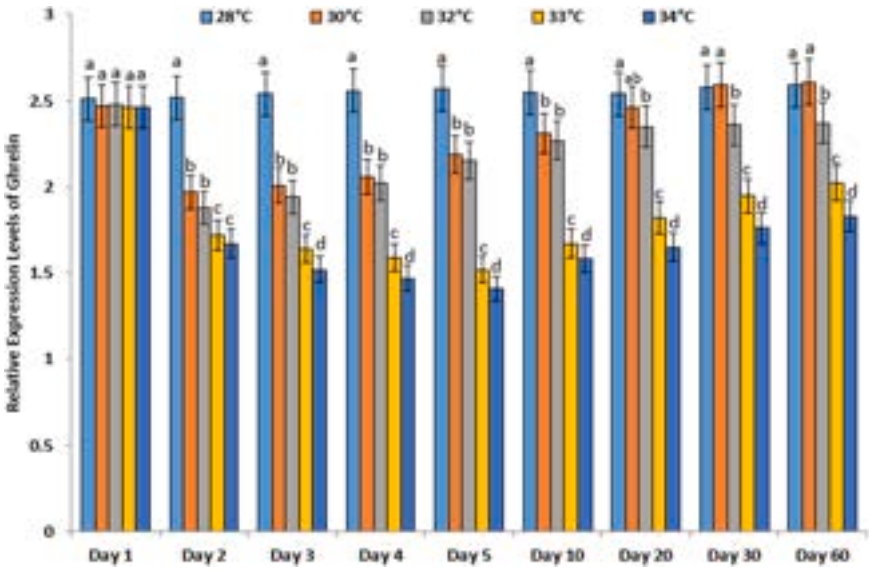

Fig. 10. Changes in relative expression levels of Ghrelin (relative to the expression of EF 1α) with increasing temperature in Rohu (Labeo rohita) juveniles across the sampling times.

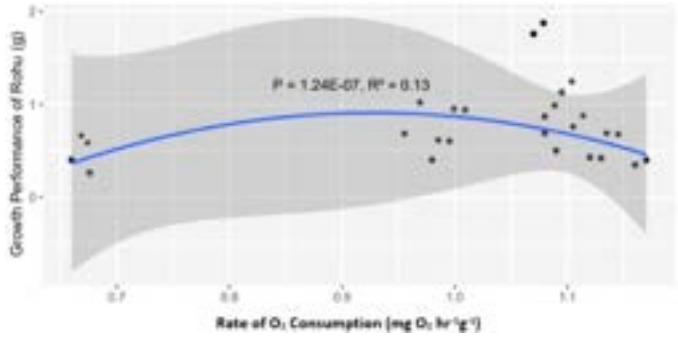

Fig. 11. : Relationship between growth and O_2 consumption rates in Rohu (*Labeo rohita*).

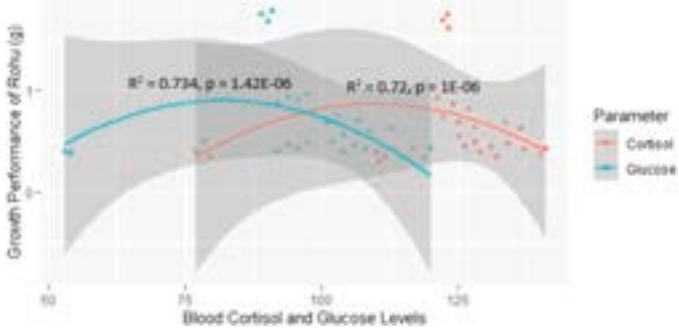

Fig. 12. : Relationship between the growth of Rohu with blood cortisol (stress hormone) and glucose levels.

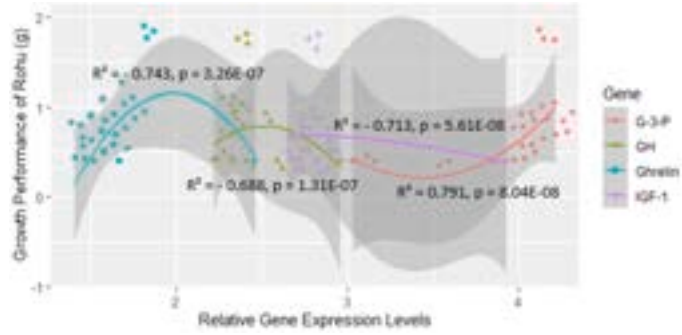

Fig. 13. Correlation pattern between growth and expression of genes associated with growth (IGF-I and GH), metabolism (G-3-P) and neuropeptide (Gherlin) controlling appetite and feed intake in Rohu.

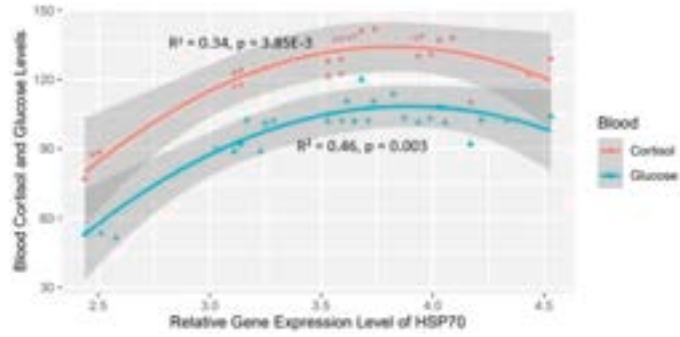

Fig. 14. Relationship between HSP70 gene expression level of Rohu with blood cortisol (stress hormone) and glucose levels.

Under stressful conditions, fish often release more cortisol and glucose by using protein stores for gluconeogenesis in the liver to eliminate or reduce the adverse effects of stressors (Wu et al., 2015; Rahi et al., 2021b; Witeska et al., 2022). Significantly higher blood cortisol and glucose levels ($P < 0.05$) of *L. rohita* under different temperature treatments across the entire experimental timeframe (Figs. 4 and 5) demonstrate the negative effects of elevated temperature. No thermal stress was induced in Rohu maintained under the control temperature (28 °C) as evidenced by the significantly lower and stable levels of cortisol and glucose levels coupled with superior growth of fish throughout the trial. Significant and positive correlations of growth with glucose and cortisol levels (Fig. 12) suggest that higher temperature treatments adversely affected fish growth. Due to the increased intensity of stress, temperature treated Rohu exhibited lower growth and survival compared to the control group. Stocking density is known to have a direct impact on fish development (Rahi et al., 2022; Rahman et al., 2022); slower growth in treatment groups (except at 30 °C) (density was

reduced in treatment groups because of increased mortality) compared to the control (no mortality resulting in a higher density) demonstrates experimental temperature induced slower fish growth in treatment groups. Higher cortisol and glucose levels in the treatment groups until the end of the experiment (on the 60th day) further support the conclusion that Rohu in different temperature treatment groups were under persistent stress.

Previous studies have shown that expression levels of specific growth regulatory (IGF-I and GH) and metabolic (G-3-P) genes play key roles in organismal growth, while the neuropeptide (ghrelin) triggers growth related processes by stimulating appetite (Triantafyllopoulos et al., 2020; Li et al., 2016; Aziz et al., 2018; Loughland and Seebacher, 2020). Several factors including different nutritional supplements, developmental stages, physiological/health status and environmental conditions, are widely recognized to directly control gene expression levels (Nadal et al., 2011; Rahi et al., 2019; Rahi et al., 2022). Therefore, any environmental stressor can potentially reduce the expression of growth genes, resulting in poor/slower growth performance (Sinha et al., 2015; Moshtaghi et al., 2017; Cossu et al., 2021; Zarantoniello et al., 2021). In the present study, experimental temperature levels significantly reduced the expression levels of GH and IGF-I genes (Figs. 6 and 7), demonstrating that higher temperatures (32 °C, 33 °C and 34 °C) had unfavorable impacts on these two growth regulatory genes in experimental Rohu. Similarly, expression of ghrelin (the neuropeptide playing important roles in food intake or appetite in fishes) was also reduced in experimental fish, indicating the adverse effect of elevated temperatures (32 °C, 33 °C and 34 °C) on the expression levels of this essential neuropeptide. Significantly negative interactions of growth with GH, IGF-I and Gherlin (Fig. 13) further validate the roles of these three genes as important growth regulators/promoters in Rohu; reduced expression of these three genes resulted in slower growth. Under stressful conditions, the metabolic activities of organisms may increase to counterbalance the adverse effects of stressors because more energy is required for stress response (Moshtaghi et al., 2018; Rahi et al., 2020). Although higher temperature treatments (32 °C, 33 °C and 34 °C) resulted in slower growth, the increased expression of the metabolic gene (G-3-P) at higher temperature treatments (Figs. 8 and 13) likely indicate the thermal stress mitigation strategy.

Heat shock protein 70 (HSP70) is a thermal stress response gene found in different fish species, showing relatively higher expression levels in response to temperature change (Sahu et al., 2015; Moshtaghi et al., 2018; Shahjahan et al., 2021; Yilmaz et al., 2021; Rahi et al., 2022). Therefore, any deviation from the control temperature potentially increases the expression levels of HSP70. During the entire study period, HSP70 expression remained significantly higher ($P < 0.05$) in *L. rohita* exposed to higher temperature treatments, demonstrating the more assertive role of temperature (Fig. 9) in altering the normal expression levels of this gene. Significant ($P < 0.05$) and positive interactions of HSP70 with blood cortisol and glucose levels of Rohu ($R^2 = 0.34$ for cortisol and $R^2 = 0.46$ for glucose) (Fig. 14) clearly indicate the intensity of thermal stress imposed on experimental fish with temperature change, as well as the effects of thermal stressor on the expression pattern of HSP70.

Optimum temperature for better growth and survival also depends on the life history stages; optimum temperature of larval stage can be different from the adults. The current study investigated the temperature effects on early stage of Rohu (≥ 0.5 g in size). Such an investigation on different developmental stages (larvae, juvenile and adults) will be more insightful in the future to obtain life stage specific optimum temperature range for maximizing production performance. Reproductive success/performance of gravid fishes also depends on temperature which is an important issue to investigate at a finer detail in the eve of climate change. This in turn, will help to predict the effects of climate change induced higher water temperature on the reproductive performance of Rohu and broadly other freshwater species.

5. Conclusion

In the current study, temperature induced physiological (growth and O_2 consumption), biochemical (blood cell counts, glucose and cortisol levels), and genetic (expression pattern of five selected genes) changes were observed in Rohu (*Labeo rohita*) fingerlings evaluated for 60 days. Changes in the experimental temperature considerably altered the measured biological parameters. Changes in the expression pattern of the evaluated genes in response to temperature changes and significant correlations in gene expression, growth, and biochemical parameters indicate critical functional roles of the genes in growth, metabolism and thermal stress response processes in Rohu. Stress mitigation measures significantly retarded growth and survival of Rohu. The stress response gene had the highest expression at 34 °C, whereas the growth genes exhibited the highest expression between 28 °C and 30 °C. Although temperature treatments imposed thermal stress at different orders of magnitude, Rohu deployed various compensatory mechanisms from different biological aspects to counteract the adverse effects of stress. As revealed in this study, Rohu can function normally at temperatures up to 32 °C without experiencing any severe impacts, while the best production performance occurring between 28 °C and 30 °C. These results are crucial for Rohu, one of Asia's most sustainably farmed freshwater carp, due to its capacity to rely on natural productivity in aquatic resources for growth while consuming less formulated feed. Therefore, from an aquaculture perspective, adequate water depth must be maintained in Rohu ponds (and typically for other freshwater species) to help maintain stable water temperature because water temperature fluctuates rapidly in shallow water ponds. The elucidation of the specific roles and functions of thermal stress related genes in Rohu opens up new avenues for future research on developing appropriate strategies for mitigating and adapting to the potential impacts of climate change on inland fisheries and aquaculture by developing temperature stress tolerant lines/strain of Rohu (broadly other freshwater aquaculture species).

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CRediT authorship contribution statement

Mridul Md. Monirul Islam: Conceptualization, Methodology, Software, Validation, Visualization, Formal analysis, Writing – original draft. **Zeehad Md. Shariar Kabir:** Methodology, Software, Validation, Visualization, Formal analysis, Writing – original draft. **Aziz Dania:** Conceptualization, Funding acquisition, Methodology, Writing – review & editing. **Salin Krishna R:** Conceptualization, Formal analysis, Writing – review & editing. **Hurwood David A:** Conceptualization, Methodology, Software, Writing – review & editing. **Rahi Md. Lifat:** Conceptualization, Data curation, Funding acquisition, Investigation, Methodology, Project administration, Resources, Supervision, Validation, Writing – review & editing.

Declaration of Competing Interest

This is to confirm that we (six authors: Md. Monirul Islam Mridul, Md. Shariar Kabir Zeehad, Dania Aziz, Krishna R Salin, David A. Hurwood and Md. Lifat Rahi) have conducted the research entitled "**Temperature Induced Biological Alterations in the Major Carp, Rohu (*Labeo rohita*): Assessing Potential Effects of Climate Change on Aquaculture Production**". This is our own research work and we do not have any conflict of interest with anybody. The authors confirm that there is no conflict of interest.

Data availability

All of the data generated for this study were analyzed and presented in Tables and Figures.

Acknowledgments

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