Research Article

Immunological, hematological, and histological responses in blood, spleen and gill of *Salmo caspius* juveniles exposed in different water temperatures

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Abstract

Caspian brown trout, *Salmo caspius,* were subject to five temperatures (5°C, 8°C, 20°C, 25°C, and 28°C) for two durations 12 and 24 hours. The fish were maintained for 48 hours at a water temperature of 9-10°C to acclimatize. The water temperature was changed gradually at a rate of 2°C per hour to the respective temperatures. Exposure to temperatures of 25 and 28°C resulted in loss of all fish. Red blood cell, hemoglobin concentration, and hematocrit were decreased at 5°C compared to control (8°C) and 20°C in duration of 12h. Temperature tension did not affect the mean cell volume and mean cell hemoglobin value during the 12 and 24h duration ($p>0.05$). Neutrophils percentage was significantly higher at 5°C and 20°C, but the most percentage of lymphocytes was noted in the control group. Cortisol, glucose, and lactate values elevated in both periods when water temperature decreased to 5°C compare to the control and 20°C groups ($p<0.05$). The levels of albumin and total protein in the duration of 12h at 20°C were significantly higher than control and 5°C, but this trend was not seen in the exposure of 24h ($p<0.05$). The lysozyme activity and immunoglobin M were affected by varying temperatures ($p<0.05$), and highest levels were observed in control groups. Most histopathological changes in gill, such as epithelial hypertrophy, curling of lamella, and necrosis of the epithelial cell were observed at 20°C; whereas these were less affected at 5°C and 8°C. Lowest hemorrhages in the spleen and lowest size of sinusoids were observed in 8°C and 5°C treatments respectively. Overall, temperature variation had a superior impact on gills than spleen. Taken together, the results of the present study showed that sudden variation beyond the optimum temperature leads to physiological and pathological changes.

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Introduction

Temperature is one of the abiotic factors that as one of the most influential and changeable agents in the environment can have a notable effect on poikilothermic vertebrates, including fish. The ability of fish to combat temperature changes varies (Cho *et al*., 2015). In the open aquaculture system, the water temperature is strictly influenced by natural factors such as rainfall, evaporation, and weather temperature (Cheng *et al*., 2013).

Gradually varying temperatures can be physiologically counterbalanced, but fast fluctuations agitate homeostasis and function as a stressor that leads to promote mitochondrial reactive oxygen species (ROS) disturb molecular processes, cellular and organism, which can trigger a decrease in physiological performance and alter the welfare of fish (Jayasundara *et al*., 2013, Almroth *et al*., 2015) and sudden variation beyond the threshold of the fish tolerance eventually result in death of the fish (Cho *et al*., 2015).

The Caspian brown trout, *Salmo caspius*, used to be one of nine subspecies of brown trout in the world, but now Caspian brown trout is classified as a species, *Salmo caspius*, that lives in Caspian Sea (Fishbase, 2020). In 2008, this species was recorded as endangered fish by International Union for Conservation of Nature (IUCN, Kiabi *et al*., 1999). Caspian brown trout is one of the valuable fishes that achieves the highest growth, weight, and size among all

brown trout (Quillet *et al*., 1992, Dorafshan *et al*., 2008). Furthermore, in 2017 more than 500,000 juvenile Caspian brown trout were delivered for stock rehabilitation in rivers leading to Caspian Sea (Roohani *et al*., 2019).

Variations in biochemical and hematological, and immune indices as a result of temperature fluctuations in some fish species is addressed (Dominguez *et al*., 2004, Lermen *et al*., 2004, Pérez-Casanova *et al*., 2008, Cho *et al*., 2015, Roychowdhury 2020). However, no information is reported on temperature oscillations in the physiological indices and the ability to regulate thermal sensibility by Caspian brown trout. In this investigation, we appraised short-term effects of the response to fluctuating water temperature on immuno-physiological, hematological, and histopathological indices in Caspian brown trout.

Material and methods

Fish sample and laboratory acclimation
A total of 150 isogenic and healthy juvenile Caspian brown trout with an average weight of 50±4.2g were taken from Shahid Bahonar Culture Center of Salmonids in Kelardasht (Mazandaran, Iran) and transferred to the trays (200×50×20cm, with mean water 100 litres) to implement the experiment in practice. Each treatment consisted of three parallel trays per each fiberglass trauph. The fish were randomly scattered with a density of 10 fish per tray. Each thermal treatment consisted of 30 fish. The fish were maintained for

48 hours at a water temperature of 9-10°C in the trays allowing fish to acclimatize to the new situation, which is the preferred Caspian brown trout temperature. To reduce metabolic effects of digestion, feeding was ceased one day before the experiment.

Experimental design
In the present study, three heat shocks (20, 25, and 28°C) and one cold shock (5°C) were designed. The exposure time of the Caspian brown trout at the mentioned temperatures was considered 12 and 24 hours. The water temperature was stable at 8±0.5°C in the control treatment tray during the study period. The temperature of each trauph gradually manipulated at a constant rate of 2°C per hour (with accuracy±0.7°C, Davis 2004; Drouillard *et al*., 2018) with thermostatically controlled heaters or a cooling coil until the respective temperature. The source of water in the current study was from well water. The fish were not fed during the investigation period. In order to minimize water temperature fluctuation, water flow was stopped. Also to supply oxygen, aeration was carried out by compressed air with air stone. Temperature and other physico-chemical parameters, such as pH, hardness, dissolved oxygen (DO), using the portable analyzer Multiline F/SET 3 and NH_3, and NO_2 were monitored during the acclimation and trial period.

During the research examination, physico-chemical factors did not fluctuate except for water temperature.

All of them were administered at the appropriate level for Caspian brown trout during the research period; DO 7.19 ± 0.5mg/L, pH: 7.35 ± 0.65, total hardness: 320.3 ± 14.3mg/L, NH_3:<0.05mg/L, and NO_2<0.01mg/L.

Blood sampling
At the end of exposure time (12 and 24h), fish were caught and euthanized with clove powder using a dosage of 300mg/L (Barij Essence, Iran, Banavreh *et al*., 2019). After sampling from the caudal vein of individual fish (3 fish per treatment) by a 2ml syringe, one aliquot from each blood sample was placed into noheparinized vacuum tube for hematological studies and another aliquot was place into heparin vacuum vial and centrifuged (3500×g for 5 min at 4°C). Samples of serum from blood were separated and stored at -20°C for future biochemical analysis.

Hematology and serum biochemistry assays
Hemoglobin concentration (Hb, g/dL), hematocrit (Hct, %) and number of red blood cells (RBCs) were appraised according to techniques explained by Blaxhall and Daisley (1973). Differential WBC, such as lymphocyte, eosinophil, monocyte, and neutrophil were manually counted using a light microscope (Nikon E 600, Tokyo, Japan). Morphometric indices of red blood cells, such as mean cell volume (MCV) and mean cell hemoglobin (MCH), were computed using the following equation:

MCV (fl): Hct (%) $\times$ 10/RBC ($\times 10^6 \mu$L)
MCH (pg): Hb (g/dL) $\times$ RBC ($10^6 \mu$L)
Concentrations of serum glucose and lactate were measured spectrophotometrically using a commercial kit (Pars Azmoon, Karaj, Iran) following the instructions of the manufacturer, and serum cortisol level was assayed by radioimmunoassay (RIA). Total protein (TP) values were appraised using biuret colorimetric method which is based on production of a violet complex among protein, and cupric ions in alkaline ambient. Concentration of serum albumin was evaluated by dye-binding among bromocresol green and albumin resulting in a colored complex.

Immune parameters

Lysozyme value was evaluated by a turbidimetric assay using the method described by Ellis (1990) determining the lytic effect of serum versus lyophilized *Micrococcus lysodeikticus* (Sigma, USA). A unit of lysozyme activity was described as the amount of serum, causing a reduction of absorbance of 0.001 per min at 22°C at 450nm. Serum immunoglobulin M (IgM) levels were measured according to the method reported by Siwicki and Anderson (1993). An aliquot of serum (0.1ml) was blended with a similar aliquot of 12.0% solution of polyethylene glycol (Sigma), and incubated for 120 min at room temperature, and centrifuged at 5000 $\times$ g for 15 min at 4°C. Afterward, the supernatant resulted from centrifuge

was diluted with 0.85% NaCl, and the protein volume was defined according to Bradford (1976) method. The discrepancy between the polyethylene glycol of the treated sample and the protein content of each untreated sample was correspondent to the total IgM value.

Gills and spleen histopathological study

At the end of the experiment, nine fish were sacrificed after anesthetizing (three fish per treatment) to remove the gills (second-gill arches of the left side) and spleen under aseptic conditions for histopathological analysis. Respective tissue samples were fixed in 10% neutral buffered formalin, and dehydrated by ethanol series of ascending concentrations, cleaning with xylene, and then paraffin embedding was conducted. The combined tissues were sectioned with a thickness of 7μm using a microtome (Leitz-1512 Germany). After that, these sections were stained with hematoxylin-eosin dye. Histopathological alterations were evaluated by a light microscope (Nikon, Ni-U, Japan).

Statistical analysis

Before the data analysis, Kolmogorov–Smirnov was employed to assess the normality of data. Two-way analysis of variance (ANOVA) was used to determine the impact of differences in temperature and duration of exposure, and the Tukey's range test was applied to evaluate significant differences among various means. All comparisons

were carried out using SPSS software version no 19.0 (SPSS, Chicago, IL, USA), and variations were recognized significant at $p<0.05$. Data were represented as mean±SD.

Results

Survival

Increasing water temperature to 25°C strictly influenced survival of Caspian brown trout ($p<0.05$), so that mortality was 100% in fish exposed to temperatures of 25 and 28°C for both 12 and 24h experiments, but one out of thirty fish (viz., 3.3%) perished in the treatment of 20°C following 24h of exposure.

Blood parameters

The results of hematological parameters are portrayed in figure 1 (a-i). RBC, Hb, and Hct were influenced by temperature fluctuation so that decreasing temperature lead to a reduction of RBC compared to control (8°C) and the group of 20°C after 12h. At 24h after exposure, this trend was reversed ($p<0.05$). The interaction between temperature and time of exposure in groups of 20°C and 5°C showed a significant difference compared with the control group ($p<0.05$). Temperature tension did not affect the MCV and MCH levels during the 12 and 24h periods ($p> 0.05$). Neutrophils percentage was significantly higher in Caspian brown trout in groups of 5°C and 20°C compared to the control group ($p<0.05$), but the highest percentage of

lymphocyte was observed in the control treatment ($p<0.05$). Exposure time did not have any effect on the lymphocyte value.

Blood biochemical indices

Blood biochemical indices are shown in figure 2 (a-e). Cortisol, glucose, and lactate levels elevated in both periods (12 and 24h) when the water temperature decreased to 5°C in comparison with the control and 20°C groups ($p<0.05$), but the difference between the control and 20°C groups was not notable ($p>0.05$). In the glucose and lactate values, the two exposure periods of 12 and 24h indicated significant differences in the group of 5°C, while the control and 20°C groups were uninfluenced by the period change. However, there was no communication between temperature and period in the glucose level. The level of albumin was highest at 20°C at 12h, but no significant difference was observed at 24h. In the control group this value was not affected by exposure time change ($p<0.05$), but this trend was not seen in other groups ($p<0.05$). On 12h sampling, increase in the concentration of total protein was recognized with increase in temperature in the 20°C group ($p<0.05$), but on 24h, no significant difference was observed in treatments ($p>0.05$).
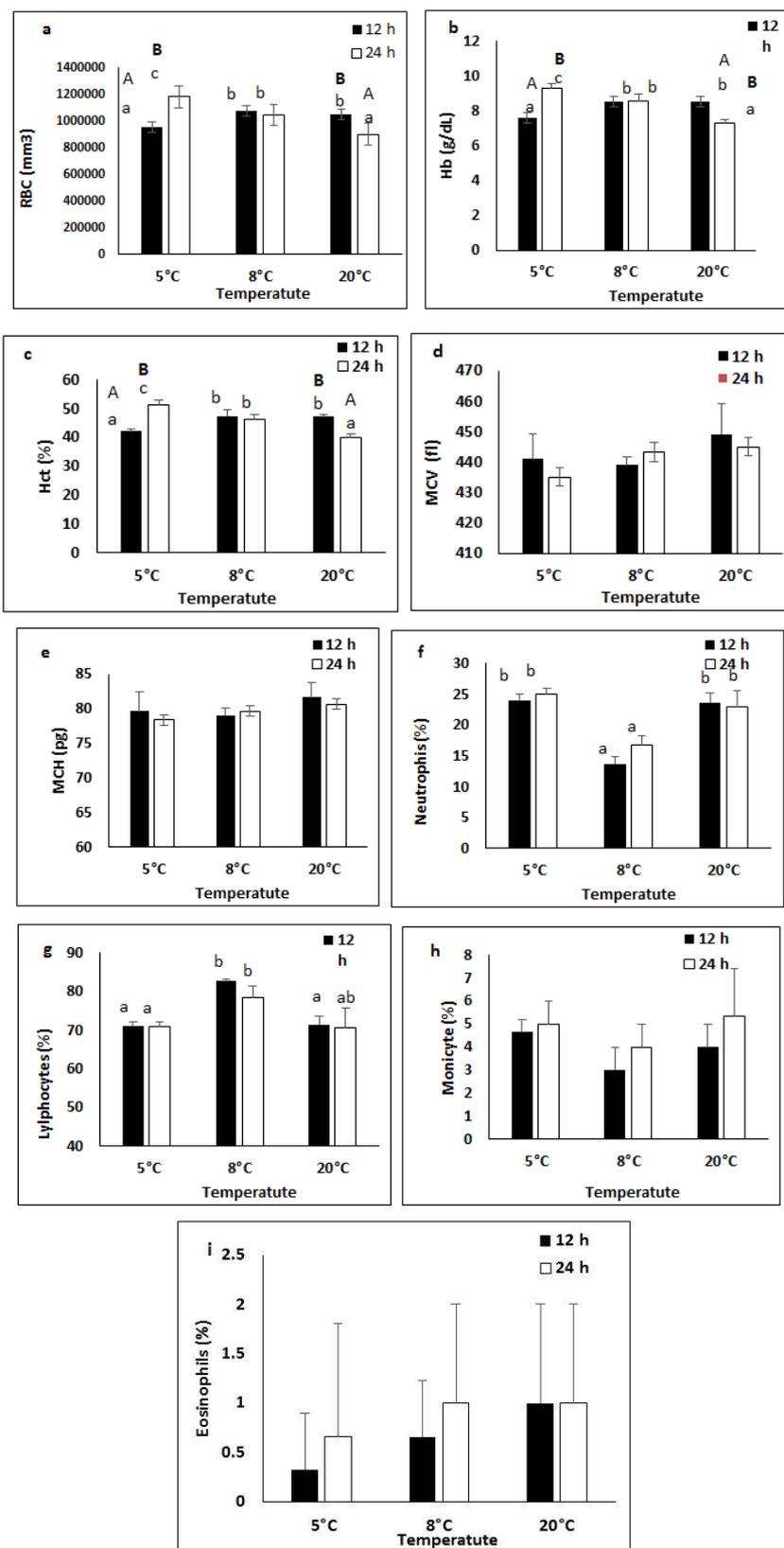
Figure 1: (a-i) Blood indices of Caspian brown trout kept at different temperatures. Different letters between groups exhibit a significant difference (*p*<0.05). The absence of letters represents no significant difference between treatments. Data are mean ±SD of the triplicated groups. Error bars show standard deviation (SD).
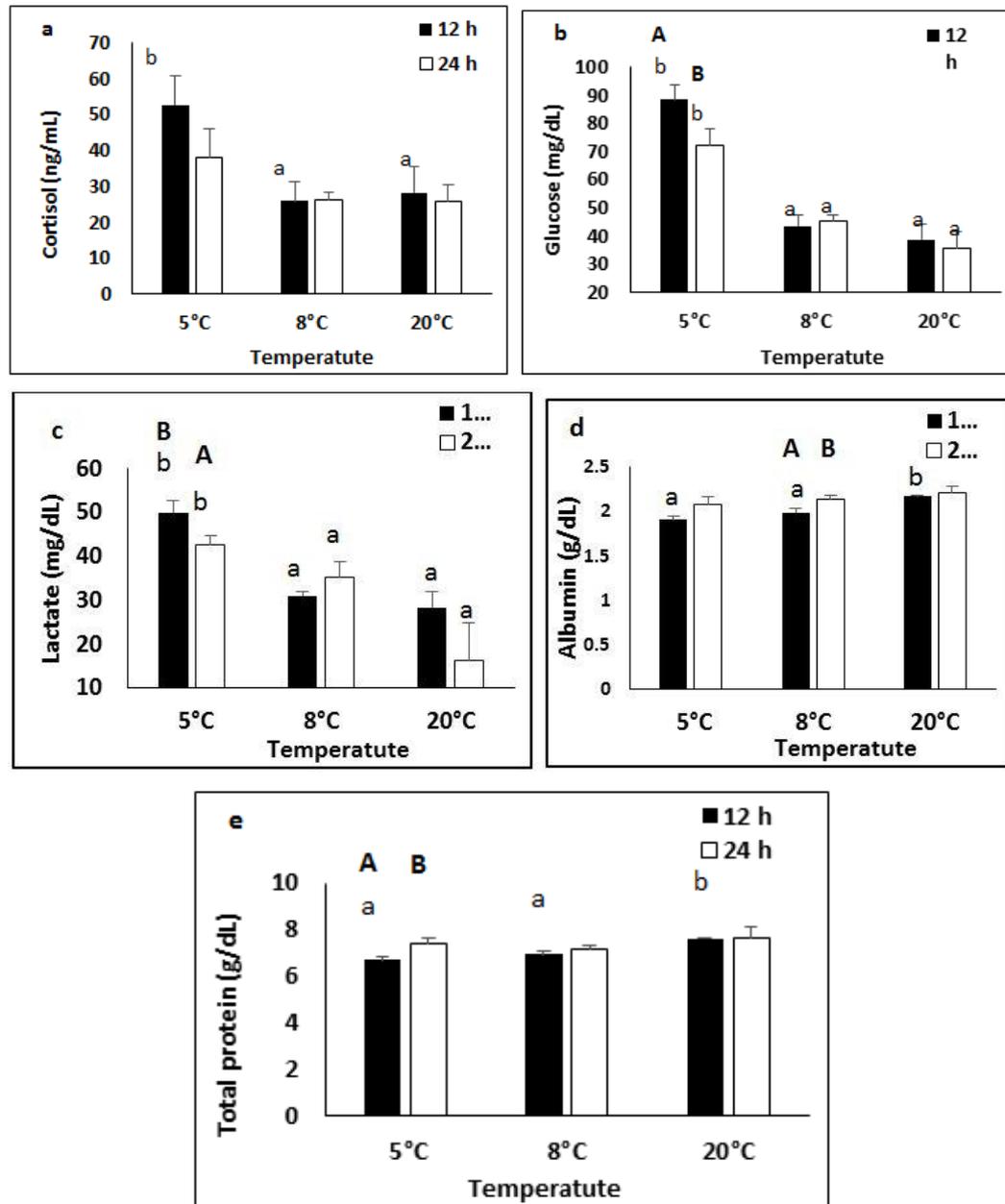
Figure 2: (a-e) Blood biochemical indices of Caspian brown trout kept at different temperatures. Different letters between groups exhibit a significant difference (*p*<0.05). The absence of letters represents no significant difference between treatments. Data are mean ±SD of the triplicated group. Error bars show standard deviation (SD).

Immunological analysis

Figure 3 shows the fluctuations of lysozyme and IgM levels after 12 and 24h exposure to oscillations of temperature conditions. There was a significant variation in the serum concentration of lysozyme between control and 5°C groups. Exposure time at 5°C had a considerable effect on lysozyme levels (*p*<0.05, Fig. 3a). The fish kept at 5°C had significantly lower IgM activity than the control group

($p<0.05$, Fig. 3b). It is worth noting, however, the IgM and lysozyme levels were not affected by the exposure time. Nonetheless, exposure time and interaction effects were significant in the control group ($p<0.05$), while other treatments showed no interaction between treatment and exposure time ($p>0.05$).
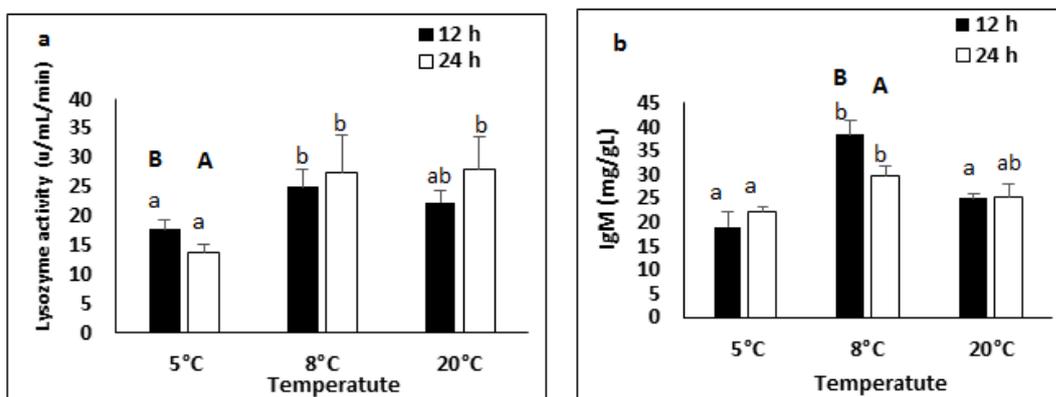

Figure 3: (a, b) Immunological indices of Caspian brown trout kept at different temperatures. Different letters between groups exhibit a significant difference ($p<0.05$). The absence of letters represents no significant difference between treatments. Data are mean ±SD of the triplicated group. Error bars show standard error.

Gill and spleen histopathology

Histopathological changes in gill and spleen are presented in Tables 1 and 2. The severity of changes in the histopathological parameters are defined as: lack of tissue lesion (-, 0-2%), mild (+, 2-10%), moderate (++, 10-40%) and severe (+++, > 40%). The curling of lamella, hemorrhage, and necrosis were moderate alterations observed in the control treatment, while in 5°C treatment, to small extent hypertrophy, lamella epithelium detachment and lamella adhesion were recognized. Severe hypertrophy (Fig. 4), curling tip of the lamella, moderate detachment of lamella epithelium, and

lamella adhesion, and eventually a mild hemorrhages were observed in 20°C treatments in comparison with the control group. The telangiectasia and hyperplasia were not witnessed in any of the treatments.

At 24h after temperature exposure, tissue changes of the spleen at different tension of temperatures are depicted in Table 2. The largest size of sinusoids was observed in the control and 20°C treatments and hemorrhages in the 5°C treatment (Fig. 5). The necrosis was not observed in any of the treatments. Also hemosiderin levels were not a discrepancy between treatments and were moderate.
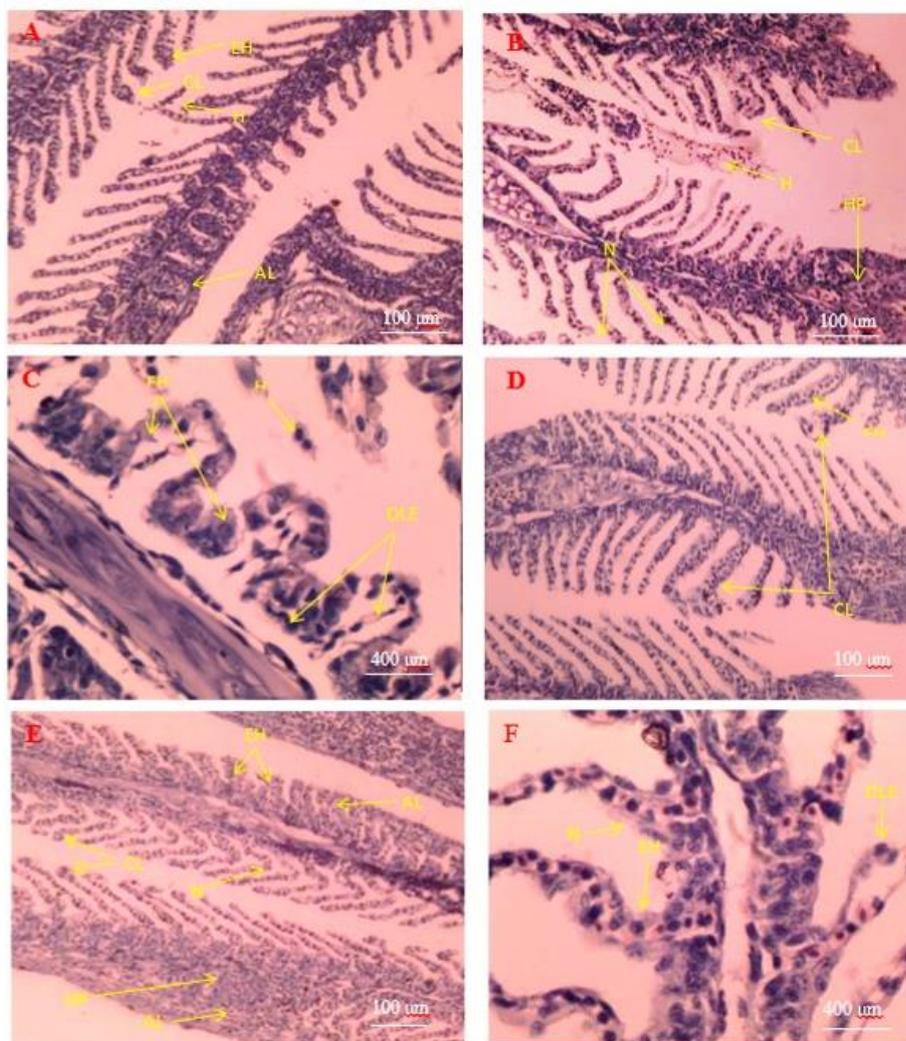
Figure 4. Longitudinal pathological section of control (8°C) temperature gill of Caspian brown trout using hematoxylin-eosin stain. (A-B), 5°C (C-D), and 20°C (E-F). CL, curling of lamella; AL, adhesion between lamellae; EH, epithelial hypertrophy; H, hemorrhage; N, necrosis of the epithelial cell; DLE, detachment of lamella epithelium; HP, hyperplasia of the epithelial cell.
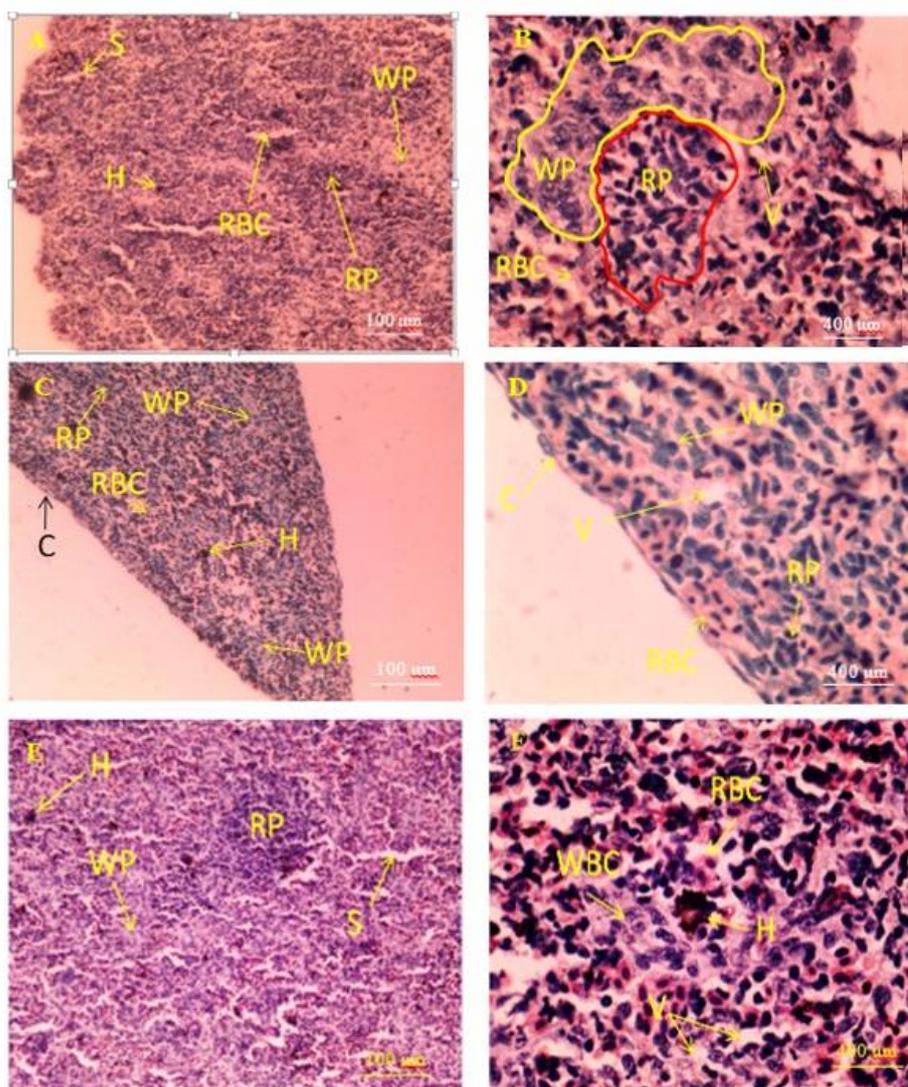
Figure 5. Longitudinal pathological section of control (8°C) temperature spleen of Caspian brown trout using hematoxylin-eosin stain. (A-B), 5°C (C-D), and 20°C (E-F). H, hemosiderin; RBC, red blood cell; RP, red pulp; S, sinusoid; V, vacuolization; WP, white pulp.

Table 1: Semi-quantitative scouring of histopathology in the gill of Caspian brown trout for 24h exposure to variant temperature. No alteration (-), mild alteration (+), moderate alteration (++), severe alteration (+++).

Symptom	Control (8°C)	5°C	20°C
Detachment of lamella epithelium	+	+	++
Hyperemia	-	-	-
Epithelial hypertrophy	+	+	+++
Hyperplasia	++	+	++
Telangiectasia	-	-	-
Curling of lamella	++	+	+++
Hemorrhages	++	+	+
Necrosis of the epithelial cell	+	-	+++
Adhesion between lamella	+	+	++

Table 2: Semi-quantitative scouring of histopathology in the spleen of Caspian brown trout for 24h exposure to variant temperature. No alteration (-), Mild alteration (+), moderate alteration (++), severe alteration (+++).

Symptom	Control (8°C)	5°C	20°C
Hemorrhages	+	++	++
Necrosis	-	-	-
Cytoplasm vacuolization	++	++	+
Hemosiderin	++	++	++
Size of sinusoid	+++	++	+++

Discussion

The present investigation announced that alteration of temperature significantly affected the survival of juvenile Caspian brown trout, especially above 20°C. The outcome of the current study showed that the temperature alterations had a tangible change in some of the hematological indices of juvenile Caspian brown trout during two exposure times. Guijarro *et al*. (2003) and De Pedro *et al*. (2005) demonstrated that RBC and Hb values of tench (*Tinca tinca*) were chiefly influenced by temperature of the water, so that above-mentioned values were reversed in relation to temperature increase. Elevated Hb and RBC at higher temperatures may be due to higher metabolic activity (De Pedro *et al*., 2005, Tirsgaard *et al*., 2015, Pinto *et al*., 2019). In low-temperature erythrocytes are not delivered to the bloodstream but to settle in the sinusoid of hepatic so that anemia was observed during low-temperature exposure (Maekawa and Kato, 2015). Findings of above mentioned authors for low temperature effects were compatible with those of our study for 12h but were completely inconsistent compared with the 24h exposure time results. Nevertheless, Mohammadi Zarejabad *et*

al. (2010) stated that RBC and Hb were not varied by diverse water temperatures. Bani and Vayghan (2011) postulated that despite the reduction of RBC in winter, promotion in MCV might exhibit the enlargement (macrocytic) of RBC, since their findings are inconsistent with ours, as there was no fluctuation in MCV and MCH that may be due to the short time of the experiment. Nevertheless, continued exposure to higher temperatures may pose numerous dysfunction of the hematopoietic organ. Leucocyte takes part directly in the fish modulator immune response, which probably describes their reinforcement immunity at optimal water temperature (Slater and Schreck, 1998, Engelsma *et al*., 2003). In our study, lymphocyte reduction under high and low-temperature stress conditions could be due to cell redistribution that is in line with Slater and Schreck (1998) and Engelsma *et al*. (2003), Biochemical parameters viz., glucose, and cortisol value in plasma, can be utilized as a general stress index in fish (Dominguez *et al*., 2004, Cho *et al*., 2015). In our study outcomes, cortisol value and carbohydrate metabolisms viz., glucose and lactate value, in low-temperature treatment were significantly higher

than those of other treatments that was inconsistent with findings of Lermen *et al*. (2004) in catfish (*Rhamdia quelen)* and Pinto *et al*. (2019) in pacu (*Piaractus mesopotamicus)* juveniles. Meanwhile, according to results of Ottolenghi *et al*. (1995) in two species of catfish (*Ictalurus meals* and *I. punctatus*) and Chen *et al*. (1996) in common carp (*Cyprinus carpio*) declared that hyperglycemia was observed in low temperature. This trend can be due to the physiological and environmental factors and differences of species. However, Chen *et al*. (1996) postulated that the glucose levels in cold water fish play an indispensable osmoregulatory role at low temperatures near freezing. Similarly, Wen *et al*. (2017) showed that due to carbohydrate balance disorders, glucose, and lactate levels were increased in discus fish (*Symphysodon aequifasciatus*) subjected to low-temperature. Levels of total protein indirectly reflect the status of specific humoral immunity (Shalaby *et al*., 2006, Banavreh *et al*., 2019, Madibana and Mlambo, 2019), while albumin as another biomolecule is reported to concern fish pathology (Madibana and Mlambo, 2019). Decreasing total protein and albumin value which was noted overtime at low temperature could be due to using these biomolecules as a terminal fuel for energy-demanding activities and energy sources. These results corroborate with similar results found in former studies (Weber and Haman, 1996, Bani and

Vayghan, 2011). Unlike the results obtained in our study, Ahmad *et al*. (2011) suggested that with an elevation of temperature (from 24°C), a significant decrease in total protein is manifested in common carp. These researchers believed that the increase of temperature might lead to malfunction of the liver that leads to loss of proteins. Previous researchers announced that IgM is influenced by the oscillation of water temperature in some of the fishes. Magnadottir *et al*. (1999) stated that the IgM value observed in Atlantic cod, *Gadus morhua* L., housed at low temperature was markedly lower. These researchers recommend that antibody response of fish is more active at higher temperature. Klesius (1990) observed no variation in the serum immunoglobulin value in Channel catfish at two different temperatures (10°C and 30°C) for 30 days. The discrepancies in the obtained results are noticeably due to differences in species, duration of exposure, variations in stress temperature and fish age.

Lysozyme is a vital non-specific immune factor (Swain *et al*., 2007, Heidari and Farzadfar 2017, Banavreh *et al*., 2019) Earlier studies by Langston *et al*., (2002) on *Hippoglossus hippoglossus*, Swain *et al*., (2007) on *Labeo rohita* and Heidari and Farzadfar (2017) on *Rutilus frissi kutum* showed that the activity of lysozyme elevated with increasing temperature, which might be one of the reasons to be predisposed to infection in lower temperature.

Variation of temperature can be a consequence of a compensatory mechanism. Following high temperature, epithelial hypertrophy and curling of lamella were extensive features. The alteration mentioned above implies the amplified risks of osmoregulatory disturbance, ion-regulatory dysfunction, and respiratory challenges in juvenile Caspian brown trout. Our findings disclosed that lower temperatures had a lesser adverse effect compared with the higher temperature. Our findings are congruent with Ahmad *et al*. (2011) that recommended high temperature lead to degenerative variation in the gill of common carp. Nonetheless, the recovery time of the fish was not investigated in the current study, and consequently, more research is warranted.

In teleosts, spleen is one of the most fundamental sources of red blood cells. Haemosiderin is one of the malfunction products of deteriorated erythrocytes. Haemosiderosis is a disordered position occurring because of the deposition of haemosiderin. Our research findings pointed out that there was no significant difference in hemosiderin, hemorrhage, and sinusoid size of the spleen of respect fish at the two exposure times. These outcomes were consistent with findings of Yifan *et al*. (2015) that proposed the spleen, like other tissues (gill, liver, and kidney), cannot exhibit significant histological lesions at different temperatures. In conclusion, the results of this work showed that water temperature fluctuations caused immune disturbances, gill, and spleen problems that lead to damaging the metabolism, anemia and growth performance.

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