Thermal stress causes nuclear and cellular abnormalities of peripheral erythrocytes in Indian major carp, rohu *Labeo rohita*

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A B S T R A C T

Rise of water temperature as a consequence of global warming is anticipated to affect the physiological activities of fish, especially in tropical regions. In the present experiment, we exposed the Indian major carp, rohu *Labeo rohita* to three different temperature regimes (30 °C as control and 33 °C and 36 °C) for 60 days and observed the effects of these temperature on: major hemato-biochemical indices (Hemoglobin; Hb, Red blood cell; RBC, White blood cell; WBC and blood glucose levels), erythrocytic nuclear abnormalities (ENA), and erythrocytic cellular abnormalities (ECA) of peripheral erythrocytes along with the formation of differential leucocytes in the blood. Fish were sacrificed at day 7, 15, 30 and 60 after the start of exposure to the temperature regimes. Hb decreased significantly on days 7 and 15 at 36 °C. Throughout the study period, the decrease of RBC and increase of WBC were significant at 36 °C. Blood glucose level increased significantly initially at day 7 but decreased significantly at day 60 at 36 °C. Frequencies of ENA (binucleated, nuclear bud, nuclear bridge, karyopyknosis and notched nuclei) and ECA (twin, fusion, echinocytic, spindle, tear drop and elongated shaped) were significantly increased at the highest temperature (36 °C) at almost all of the sampling days. In the case of differential leucocyte count, high temperature caused a significant increase in the number of neutrophils and a significant decrease in the number of lymphocytes. Overall, these results indicate that chronic exposure to high temperature (36 °C) induces a number of stress responses in rohu and that temperature should be kept below 36 °C in the aquaculture setting to avoid damage to the fish.

1. Introduction

Different physiological activities in fish are greatly influenced by water temperature. Increase of water temperature due to anthropogenic activities and climatic change is expected to affect the distribution and abundance of aquatic cold-blooded animals (Portner and Farrell, 2008; Shahjahan et al., 2013; Verhille et al., 2016), especially tropical species due to the higher route of change in these environments and thermally stable climate in which these organisms have developed (Deutsch et al., 2008; Burrows et al., 2011; Tewksbury et al., 2008). Typically, in the tropical regions, fishes are adapted to water temperature ranges of between 25 and 35 °C (Howerton, 2001). It has been reported that temperature can reach to level that may affect the growth and normal physiological processes of fish (Portner et al., 2001; Fu et al., 2018). Therefore, climate change is predicted to affect the behavior and physiology of aquatic animals along with their ecology (Walther et al., 2002).

Since most poikilothermic animals, such as fish, cannot regulate the internal body temperature, environmental temperature plays a major role in regulating the overall performances of the organisms as a representative of physical wellbeing (Angilletta, 2009). The effects of temperature changes on fish species may be predicted through physiological studies (Somero, 2010). Almost all biochemical and physiological activity is greatly affected by rising water temperature, causing stress and alteration of blood chemistry standards because fish are aquatic poikilothermic animals. Chatterjee et al. (2004) stated that high temperature increased the chemical reactions in the bodies of fish and greatly affected the physiological process when exceeded the level of tolerance.

Hemato-biochemical parameters are a common endpoint measured for assessing the physiological status of fish exposed to different environmental stressors, including temperature (Mattsson et al., 2001; Affonso et al., 2002; Cazenave et al., 2009; Elahee and Bhagwant, 2006;

Sharmin et al., 2015; Salam et al., 2015). Fish erythrocytes possess hemoglobin that transports oxygen to all tissues, hence it bears the probable cues for any abnormal level and pathological indications. Therefore, blood hemoglobin contents are frequently used to assess anemia and physiological well-being of fish (Hesser, 2011). Fluctuations in temperature are known to alter the size and number of erythrocytes and affect hemoglobin concentration (Allen, 1993; Hlavova, 1993; Ytrestoyl et al., 2001). The rise in environmental temperature reduces the dissolved oxygen content in the water which in turn increases fish metabolism. Fish adjust to this adverse environmental condition by raising their total hemoglobin level (Brix et al., 2004). Different morphological (cellular and nuclear) abnormalities of erythrocytes and differential leucocytes count are also very important analytical factors to assess the stress caused by any environmental variations (Ghaffar et al., 2015; Shahjahan et al., 2018), but to date, little has been explored in these important factors.

Although a number of experiments were conducted to assess the thermal stress response on different aquatic poikilotherms (Bevelhimer and Bennett, 2000; Shahjahan et al., 2018; Islam et al., 2019), effects of chronic exposure to high temperature on Indian major carp (*L. rohita*) has so far not been studied. The Indian major carp (locally known as rohu) is the most commonly cultured freshwater fish species in Bangladesh. This species is mostly abundant in Bangladesh, northern and central India, Myanmar, Nepal and Pakistan (Talwar and Jhingran, 1991). It is considered as one of the most important farmed fish because of its high growth potential, nutritious and delicious attributes and high market value (Dahanukar, 2010). Its fry and fingerlings are easily available for culture and traits preferred by the consumers have made the species a suitable aquaculture candidate in Bangladesh and other countries of its distribution. Furthermore, it is an ideal species for carp polyculture and can be stocked with other carps like catla (*Catla catla*) and mrigal (*Cirrhinus mrigala*). The purpose of the present study was to assess how chronic exposure to high temperature alters hemato-biochemical parameters, and cellular and nuclear structures of erythrocytes in rohu.

2. Materials and methods

2.1. Experimental animal

Healthy, disease free rohu, *L. rohita* fry were collected from Bangladesh Fisheries Research Institute (BFRI) Freshwater Station, Mymensingh. Fish had an average length and weight of 11.56 ± 0.42 cm and 15.96 ± 0.70 g, respectively. The fish were reared in reinforced glass aquaria holding 100 L of water for 15 days at 30 ± 0.5 °C in a controlled environment before starting the experiment. The fish was fed a commercial diet (manufactured by Mega Fish *Feeds* Ltd., Bangladesh) twice a day *ad libitium*.

2.2. Design of experiment

To conduct the experiment, nine reinforced glass aquaria (75 cm × 45 cm × 45 cm each) were cleaned and rinsed with clean water and set up in the Wet Laboratory of the Faculty of Fisheries, Bangladesh Agricultural University. The aquaria were then filled with 100 L of clean tap water and stocked with 20 fry per aquarium. The aquaria were provided with filtration cum aeration device (Sebo-aquarium Internal Filter WP-850F, Yiwu Nihao Aquarium, Zhejiang, China) for self-cleaning and aeration throughout the study period. The fish were supplied with commercial diet twice a day *ad libitium*. Water temperature was gradually increased from 30 °C (control) by 1 °C/day for the adaptation of fish towards the target temperature of 33 °C and 36 °C for each treatment having three replicates. The fish was then reared in 30, 33 and 36 °C for 60 days. The target temperature was achieved with the thermostat (REI-SEA, 300 W, Japan). We followed the guidance approved by the Animal Welfare and Ethical Committee of Bangladesh

Agricultural University.

2.3. Blood sampling

Six fish (n = 6) were sampled from each temperature regime after 7, 15, 30 and 60 days of exposure to experimental temperature conditions. Fish were anesthetized using clove oil at the rate of 5 mg/L and blood samples were promptly collected from the caudal vein using heparinized plastic syringe to avoid the consequence of stress and blood coagulation. The blood samples were stored in sterilized microfuge tubes containing 20 mM EDTA to retard further coagulation. During laboratory analysis, any unexpected blood clotting was minimized by gentle shaking the microfuge tube.

2.4. Measurement of hemato-biochemical parameters

Hemoglobin (g/dL) was measured with hemoglobin strips using a digital EasyMate® GHb (Model: ET 232, Hb/Glu double monitoring system, Bioptic technology Inc. Taiwan 35057) immediately following blood sample collection. Red blood cells (RBCs) and White blood cells (WBC) were counted using Neubauer hemocytometer (Blaxhall and Daisley) under a light microscope. Blood glucose (mg/dL) was measured by glucose strips using a digital EasyMate® GHb (Model: ET 232, Hb/Glu double monitoring system) immediately following blood sample collection.

2.5. Erythrocytic cellular abnormalities (ECA), erythrocytic nuclear abnormalities (ENA) and differential leucocytes

Each blood sample was smeared onto a clean microscopic slide immediately following blood sample collection. The slide was air dried for 10 min and then fixed with methanol for further 10 min. Finally the slides were stained with 5% Giemsa stain, rinsed with distilled water and kept overnight for subsequent air drying. The slides were then mounted with dibutylphthalate polystyrene xylene (DPX). From each fish, three slides were prepared and from each slide 2000 cells were scored and at least three fish were analyzed from each temperature group. During scoring, only cells having an intact cellular and nuclear membrane were considered. To investigate ENA, ECA and differential leucocytes associated with different temperature regimes, the slides were examined under an Optica optical microscope (G-206, Italy) with 100 × objective lens.

Erythrocytic nuclear abnormalities (ENA) resulting from different temperature treatments were categorized according to Carrasco et al. (1990). For instance, cells having two nuclei were regarded as binuclei, cells having nuclei with evagination (something like a bud) were considered as nuclear bud, and individual nuclei connected by a thin strand were considered as nuclear bridge. Cells that did not contain nuclear material but rather had vacuoles in the nuclei were recorded as notched nuclei. Cells with condensation and clumping of the chromatin materials in the periphery of the nuclei along with irregular nuclear membranes were characterized as karyopyknosis.

Erythrocytic cellular abnormalities (ECA) are those that are unlike the regular oval shaped erythrocyte with a concentrated nucleus. ECA were categorized as two cells joined together by the surface of the cell to form twin; combining of more than two cells to form denser/thicker was considered fusion. Spiculated RBCs having many small uniform surface projections were characterized as echinocytes. Cells that are shaped like spindles, being more or less round in the middle with two pointed ends are spindles. Tear drop cells that has slightly rounded or blunted ends or having sharp points in smeared preparation. Cell that had an unusual length when compared to normal cells were designated as elongated cells.

Different leucocytes namely monocytes, neutrophils, lymphocytes and eosinophils were observed and their frequencies of occurrence were counted from smeared slides.

2.6. Water quality parameters

Water quality parameters such as dissolved oxygen (mg/L), free CO_2 (mg/L), pH and total alkalinity (mg/L) were measured at every sampling day over the experimental period. Dissolved oxygen (mg/L) was measured with a DO meter (Model DO5509, Lutron, made in Taiwan) and pH using a portable pH meter (Model RI 02895, HANNA Instruments Co.) by directly inserting the probe into the aquaria. The sample water was collected and brought to the nearby Laboratory of Water Quality for analyzing free CO_2 and total alkalinity. Free CO_2 (mg/L) was monitored using phenolphthalein indicator and 0.0227N NaOH titrant, and the total alkalinity (mg/L) was measured by titrimetric method using methyl orange indicator and 0.02N H_2SO_4 titrant.

2.7. Statistical analyses

All the values were represented as mean ± standard deviation. To test the statistically significant difference among the different temperature conditions, one-way analysis of variance (ANOVA) was carried out followed by Tukey's post hoc test. Mann-Whitney *U* test with a Bonferroni correction was used to assess the significant difference among the days of exposure to different temperature treatments. We set statistical level of significance at $p < 0.05$. Statistical analyses ware carried out using SPSS 14.0 for Windows (SPSS Inc., Chicago, IL).

3. Results

3.1. Changes in hemato-biochemical parameters exposed to different temperature conditions

Hematological parameters, such as Hb level (g/dL), number of RBCs ($\times 10^6/mm^3$) and WBCs ($\times 10^3/mm^3$), and biochemical parameter blood glucose level (mg/dL) were measured at 7, 15, 30 and 60 days of exposure in the three different temperature conditions.

At days 7 and 15, significant ($p < 0.05$) decreases in the values of Hb content of fish blood were observed at 33 °C and 36 °C when compared with the blood of fish reared at 30 °C. However, no distinct changes in the values of Hb of fish blood were observed at days 30 and 60 among the treatments (Fig. 1A). At day 7, the number of RBCs ($\times 10^6/mm^3$) in fish blood was significantly ($p < 0.05$) lower at 33 °C and 36 °C compared to the fish kept at 30 °C. Whereas at days 15, 30 and 60, the number of RBC was significantly ($p < 0.05$) lower when fish were reared only at 36 °C compared to 30 °C (Fig. 1B). In contrast, the number of WBCs present in the fish blood was significantly ($p < 0.05$) higher at 36 °C compared to that of the fish kept at 30 °C in all four sampling days (Fig. 1C).

Blood glucose (mg/dL) level significantly ($p < 0.05$) increased at day 7 at 36 °C compared to 30 °C, and showed no distinct changes at days 15 and 30, but significantly ($p < 0.05$) decreased at day 60 at 36 °C (Fig. 1D). At days 15, 30 and 60, blood glucose levels were significantly lower than that was recorded at day 7 at the highest temperature (36 °C).

3.2. Frequencies of erythrocytic nuclear abnormalities (ENA) caused by high temperature
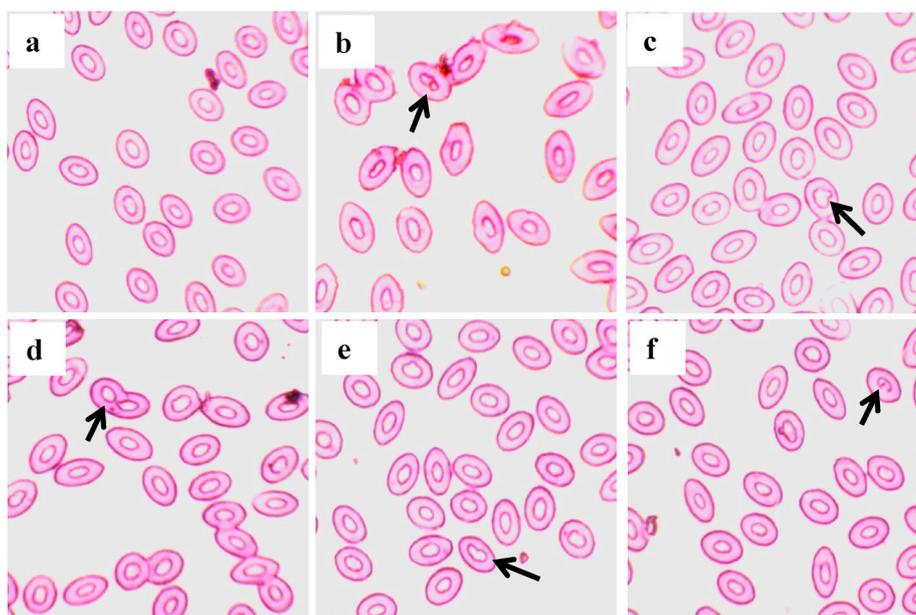
Different types of erythrocytic nuclear abnormalities (ENA) like binuclei, nuclear bud, nuclear bridge, karyopyknosis and notched nuclei were observed in the different temperature treatments at different sampling dates (Fig. 2). The frequencies of different ENA are presented in Table 1. Though the frequencies of different ENA increased significantly in 33 °C at day 7, whereas a significant ($p < 0.05$) increase in the frequencies of ENA in 36 °C treated groups was observed in comparison to 30 °C treated group throughout the experimental periods.
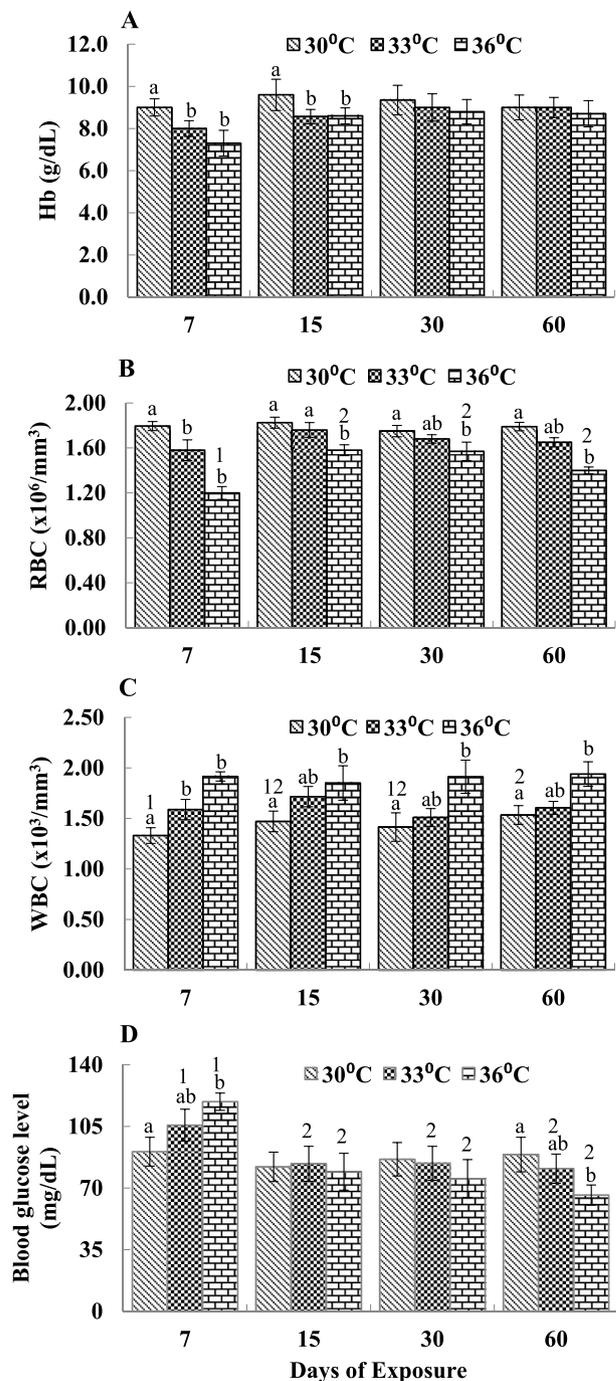
Fig. 1. Changes in hemato-biochemical parameters of *L. rohita* after exposure to different temperature - A. hemoglobin levels (g/dL); B. number of RBC ($\times 10^6/mm^3$); C. number of WBC ($\times 10^3/mm^3$); and D. blood glucose levels (mg/dL). Values accompanied by different alphabets are indicating statistically significantly different ($p < 0.05$) among treatments. Values with different numeric superscripts differ significantly ($p < 0.05$) among days of exposure. All values expressed as mean ± SD (n = 6).

3.3. Frequencies of erythrocytic cellular abnormalities (ECA) caused by high temperature

Various types of erythrocytic cellular abnormalities (ECA) such as twin, fusion, echinocytic, spindle, tear-drop and elongated shaped cells were observed in the different temperature treatments at different sampling dates (Fig. 3). Frequencies of several types of erythrocytic cellular abnormalities (ECA) in fish exposed to different temperature

Fig. 2. Various erythrocytic nuclear abnormalities (ENA) in giemsa stained blood smears of *L. rohita* treated with three different temperature conditions; (a) regular cells, (b) binucleated, (c) nuclear bud, (d) nuclear bridge, (e) karyopyknosis, and (f) notched nuclei.

Table 1
Frequencies of erythrocytic nuclear abnormalities (ENA) in *L. rohita* after exposure to three different temperature conditions.

ENA	Temperature (ºC)	Percentage of ENA			
		Exposure time (day)			
		7	15	30	60
Binuclei	30	0.28 ± 0.05^a	0.25 ± 0.0^a	0.21 ± 0.01^a	0.27 ± 0.01
	33	$0.83 \pm 0.09^{b,1}$	$0.45 \pm 0.05^{ab,2}$	$0.31 \pm 0.03^{ab,2}$	0.28 ± 0.01^2
	36	$0.95 \pm 0.13^{b,1}$	$0.78 \pm 0.07^{b,12}$	$0.59 \pm 0.05^{b,2}$	0.42 ± 0.03^2
Nuclear bud	30	0.35 ± 0.03^a	0.28 ± 0.04^a	0.22 ± 0.02^a	0.30 ± 0.01^a
	33	$1.48 \pm 0.07^{b,1}$	$0.63 \pm 0.04^{ab,2}$	$0.45 \pm 0.03^{ab,2}$	$0.31 \pm 0.02^{ab,2}$
	36	$1.56 \pm 0.09^{b,1}$	$0.77 \pm 0.05^{b,2}$	$0.66 \pm 0.03^{b,2}$	$0.61 \pm 0.03^{b,2}$
Nuclear bridge	30	0.29 ± 0.03^a	0.23 ± 0.01^a	0.21 ± 0.01^a	0.20 ± 0.01^a
	33	$0.85 \pm 0.09^{b,1}$	$0.51 \pm 0.05^{b,12}$	$0.38 \pm 0.05^{ab,2}$	$0.33 \pm 0.03^{ab,2}$
	36	$1.10 \pm 0.13^{b,1}$	$0.63 \pm 0.05^{b,2}$	$0.66 \pm 0.05^{b,2}$	$0.69 \pm 0.05^{b,2}$
Karyopyknosis	30	0.73 ± 0.09^a	0.68 ± 0.07	0.61 ± 0.05	0.66 ± 0.07
	33	1.16 ± 0.15^{ab}	0.98 ± 0.11	0.83 ± 0.09	0.88 ± 0.09
	36	$1.63 \pm 0.15^{b,1}$	0.73 ± 0.09^2	0.66 ± 0.05^2	0.76 ± 0.11^2
Notched nuclei	30	0.57 ± 0.09^a	0.56 ± 0.05^a	0.46 ± 0.07	0.48 ± 0.05
	33	$1.20 \pm 0.13^{b,1}$	$0.79 \pm 0.09^{ab,2}$	0.62 ± 0.10^2	0.63 ± 0.09^2
	36	$1.66 \pm 0.15^{b,1}$	$0.93 \pm 0.11^{b,2}$	0.72 ± 0.11^2	0.61 ± 0.09^2

Values of a single ENA in a column with different alphabetical superscripts are significantly ($p < 0.05$) different. Values with different numeric superscripts in a row differ significantly ($p < 0.05$) among days of exposure. All values expressed as Mean ± SD. Three slides were prepared from each fish and 2000 cells were scored from each slide and three fish were analyzed from each group.

conditions are presented in Table 2. A significant increase ($p < 0.05$) in ECA was found in the blood of fishes exposed to the highest temperature regime (36 °C) similar to that was observed in the case of ENA.

3.4. Differential leucocytes count at different temperature conditions

We observed different counts of leucocytes such as monocytes, neutrophils, lymphocytes and eosinophils from the different treatment groups (Fig. 4). Frequencies of different leucocytes in the blood of fishes exposed to different temperature regimes are shown in Table 3. Opposite results were observed in the case of neutrophil and lymphocyte counts with a significant ($p < 0.05$) increase in the number of neutrophils and a significant ($p < 0.05$) decrease in the number of lymphocytes observed in fish treated at the highest temperature regime (36 °C).

3.5. Effects of high temperature on water quality parameters

Dissolved oxygen (mg/L), free CO_2 (mg/L), pH and total alkalinity (mg/L) measured during the experimental period are presented in Table 4. Dissolved oxygen (mg/L) decreased significantly with increasing water temperature, whilst free CO_2 (mg/L) showed a significant increase with increasing temperature ($p < 0.05$). No significant change was observed in the values of pH and total alkalinity throughout the study period irrespective of the temperature conditions (Table 4).

4. Discussion

Environmental stressors, including temperature are known to affect various physiological processes in fish. Although fish showed no mortality in the three different temperature regimes in this study, we observed significant temperature induced changes in hemato-
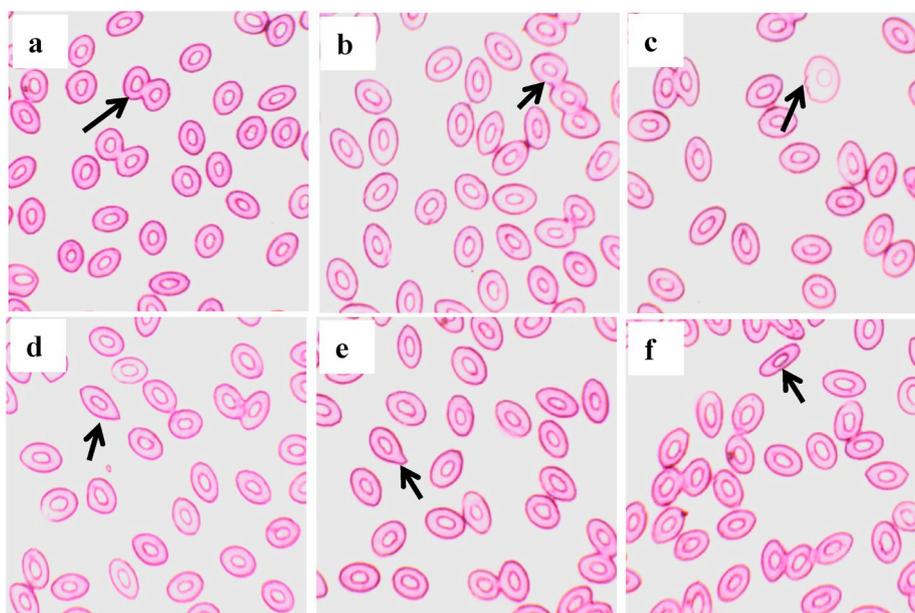
Fig. 3. Various erythrocytic cellular abnormalities (ECA) in giemsa stained blood smears of *L. rohita* treated with three different temperature conditions; (a) twin, (b) fusion, (c) echinocytic, (d) spindle, (e) tear drop shaped and (f) elongated shape.

Table 2
Frequencies of erythrocytic cellular abnormalities (ECA) in *L. rohita* after exposure to three different temperature conditions.

ECA	Temperature (ºC)	Percentage of ECA			
		Exposure time (day)			
		7	15	30	60
Twin	30	0.55 ± 0.07^a	0.58 ± 0.05^a	0.51 ± 0.03^a	0.53 ± 0.03
	33	$1.53 \pm 0.013^{b,1}$	$0.83 \pm 0.07^{ab,2}$	$0.84 \pm 0.09^{ab,2}$	0.80 ± 0.07^2
	36	$1.66 \pm 0.15^{b,1}$	$1.62 \pm 0.09^{b,1}$	$0.96 \pm 0.11^{b,2}$	0.71 ± 0.13^2
Fusion	30	0.81 ± 0.09^a	0.78 ± 0.09^a	0.80 ± 0.09	0.79 ± 0.09
	33	$1.51 \pm 0.13^{b,1}$	$0.94 \pm 0.11^{ab,2}$	0.83 ± 0.10^2	0.78 ± 0.09^2
	36	$1.53 \pm 0.15^{b,1}$	$1.33 \pm 0.11^{b,1}$	1.04 ± 0.09^2	0.71 ± 0.07^2
Echinocytic	30	0.45 ± 0.13^a	0.35 ± 0.09^a	0.40 ± 0.07^a	0.38 ± 0.05^a
	33	$1.23 \pm 0.09^{b,1}$	$0.91 \pm 0.05^{ab,2}$	$0.73 \pm 0.05^{ab,2}$	$0.76 \pm 0.03^{ab,2}$
	36	1.60 ± 0.10^b	$1.03 \pm 0.09^{b,2}$	$0.98 \pm 0.05^{b,2}$	$0.86 \pm 0.05^{b,2}$
Spindle	30	0.71 ± 0.11^a	0.73 ± 0.09^a	0.68 ± 0.07^a	0.77 ± 0.09
	33	$1.63 \pm 0.15^{b,1}$	$1.08 \pm 0.09^{ab,2}$	$0.80 \pm 0.07^{ab,2}$	0.83 ± 0.07^2
	36	$1.66 \pm 0.18^{b,1}$	$1.38 \pm 0.07^{b,12}$	$0.96 \pm 0.07^{b,2}$	0.80 ± 0.05^2
Tear-drop	30	0.60 ± 0.07^a	0.64 ± 0.05^a	0.63 ± 0.09	0.56 ± 0.07
	33	$1.43 \pm 0.11^{b,1}$	$0.98 \pm 0.09^{ab,12}$	0.78 ± 0.07^2	0.73 ± 0.07^2
	36	$1.56 \pm 0.11^{b,1}$	$1.38 \pm 0.09^{b,12}$	0.90 ± 0.10^2	0.84 ± 0.10^2
Elongated	30	0.58 ± 0.07^a	0.57 ± 0.05^a	0.56 ± 0.05^a	0.55 ± 0.07^a
	33	$1.26 \pm 0.13^{b,1}$	$1.04 \pm 0.09^{b,12}$	$0.80 \pm 0.09^{ab,2}$	$0.81 \pm 0.09^{ab,2}$
	36	$1.67 \pm 0.15^{b,1}$	$1.50 \pm 0.09^{b,12}$	$1.28 \pm 0.07^{b,2}$	$0.92 \pm 0.07^{b,2}$

Values of a single ECA in a column with different alphabetical superscripts are significantly ($p < 0.05$) different. Values with different numeric superscripts in a row differ significantly ($p < 0.05$) among days of exposure. All values expressed as Mean $\pm$ SD. Three slides were prepared from each fish and 2000 cells were scored from each slide and three fish were analyzed from each group.

biochemical indices along with morphological alterations of erythrocytes and leucocytes, caused by chronic exposure of fish to high temperature.

It has been reported that stress causes alteration of hematological parameters and thus affects the overall state of fish physiology (Beyea et al., 2005; Shahjahan et al., 2018). The significant decrease in Hb and RBC content in this study might be due to stress produced by exposure to high temperature and subsequent failure of the hematopoietic system. The stress response of fish to high temperature, as observed in earlier studies, is species specific and depends mostly on exposure time and adaptability of the species. For example, Hb content was shown to increase at high temperature in tilapia, but remained constant in common carp and trout (Smit et al., 1981). Exposure to elevated temperatures, causing thermal stress and altering the Hb and RBC content in the

Neotropical fish, *Prochilodus scrofa* (Carvalho and Fernandes, 2006) is in agreement with this study. Recently, a decrease in the value of Hb and RBC were also observed after exposure to high temperature in the striped catfish, *Pangasianodon hypophthalmus* (Shahjahan et al., 2018; Islam et al., 2019).

Blood smears from fish exposed to the high temperature regime revealed a significant increase in the frequencies of several ENA and ECA, believed to occur by changing the physical properties of plasma membranes of fish erythrocytes. It has been reported that changes in temperature alters the lipid constituents of blood cells as well as changing membrane fluidity, micro-surrounding of proteins and protein-lipid interactions of erythrocytes in poikilo-thermal animals (Kreps, 1981; Dey et al., 1993; Avrova, 1999). Moreover, increase of lipid peroxidation products in erythrocytes of fish exposed to high
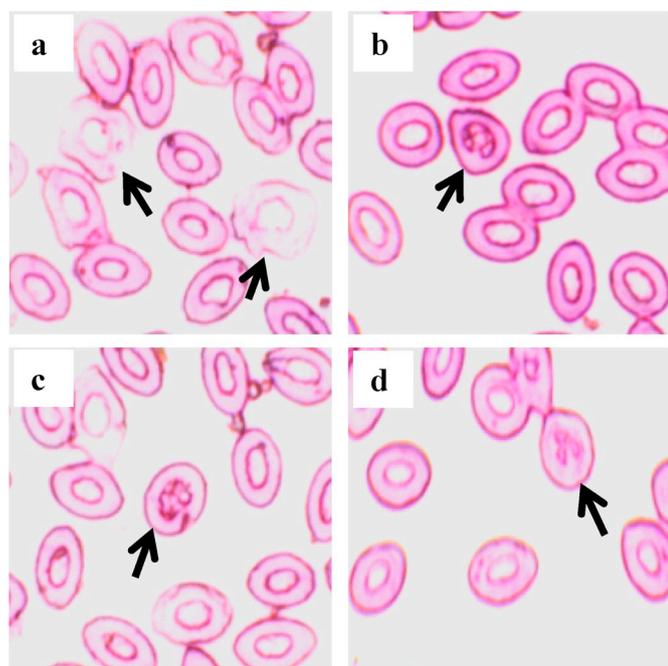
Fig. 4. Different leucocyte in giemsa stained blood smears of *L. rohita* treated with three different temperature conditions; (a) monocytes, (b) neutrophils, (c) lymphocytes and (d) eosinophils.

temperature might cause erythrocytic cellular abnormalities like elongated, fusion, tear-drop and twin cells (Bai et al., 2014; Ghaffar et al., 2015). Likewise, cellular abnormality, named echinocytic, happens due to interruption of the solubility of lipid in the erythrocyte membrane that finally results in apoptosis (Walia et al., 2013). In addition to this type of ECA, morphological changes in the plasma membrane also affect surface deformability and as a result erythrocyte becomes more vulnerable to burst when crossing small capillaries. Therefore, the mechanism that regulates the stability of erythrocyte membrane structure during thermal adaptation depends on alternation of the intra- and inter-molecular interaction and cytoskeleton protein interactions excluding the regulation of lipid composition.

In the present study, we observed a significant increase in the values of WBC in the higher temperature regime (36 °C) throughout the study period. The increase in the number of WBCs might be caused by the increase of antibody production (Raphael and Kuttan, 2003) which helped the organism in survival and healing during exposure to toxic pollutants (Joshi et al., 2002; Begg and Pankhurst, 2004). A significant increase in the number of neutrophils and decrease in the number of lymphocytes were observed in the present study at the highest temperature (36 °C) throughout the study period. Similar results were observed in earlier studies, where increases in the number of neutrophils and decreases the number of lymphocytes were observed due to stress in channel catfish, *Ictalurus punctatus* (Ainsworth et al., 1991) and in boars, *Sus scrofa* (Bilandzic et al., 2006). As the numbers of neutrophils and lymphocytes are reported to be altered/influenced by stress in counter direction (Davis et al., 2008), it can be inferred that the high temperature (36 °C) caused stress to rohu in the present study.

Table 3
Differential leucocyte count in *L. rohita* after exposure to three different temperature conditions.

Different leucocytes	Temperature (°C)	Percentage of different leucocytes			
		Exposure time (day)			
		7	15	30	60
Monocytes	30	2.0 ± 1.0	2.0 ± 1.0	2.0 ± 1.0	2.0 ± 1.0
	33	3.0 ± 1.0	3.0 ± 1.0	3.0 ± 1.0	3.0 ± 1.0
	36	2.0 ± 1.0	2.0 ± 1.0	2.0 ± 1.0	2.0 ± 1.0
Neutrophil	30	15.0 ± 1.0^a	17.0 ± 1.0^a	19.0 ± 1.0^a	16.0 ± 1.0^a
	33	22.0 ± 3.0^{ab}	23.0 ± 3.0^{ab}	22.0 ± 3.0^{ab}	22.0 ± 3.0^{ab}
	36	70.0 ± 5.0^b	67.0 ± 5.0^b	63.0 ± 5.0^b	51.0 ± 5.0^b
Lymphocytes	30	18.0 ± 1.0^b	19.0 ± 1.0^b	18.0 ± 1.0^b	16.0 ± 1.0^b
	33	13.0 ± 4.0^{ab}	11.0 ± 4.0^{ab}	12.0 ± 4.0^{ab}	14.0 ± 4.0^{ab}
	36	8.0 ± 1.0^a	9.0 ± 1.0^a	8.0 ± 1.0^a	9.0 ± 1.0^a
Eosinophil	30	1.0 ± 0.0	1.0 ± 0.0	2.0 ± 0.0	1.0 ± 0.0
	33	1.0 ± 0.0	1.0 ± 0.0	1.0 ± 0.0	2.0 ± 0.0
	36	2.0 ± 1.0	2.0 ± 1.0	1.0 ± 1.0	1.0 ± 1.0

Values with different alphabetical superscripts in a column differ significantly ($p < 0.05$) among different temperature in differential leucocytes. All values expressed as mean ± SD. Three slides were prepared from each fish and 200 cells were scored from each slide and three fish were analyzed from each group.

Table 4
Water quality parameters (Mean ± SD) during the study periods.

Parameter	Temperature (°C)	Days of exposure			
		7	15	30	60
Dissolved oxygen (mg/L)	30	7.2 ± 0.56^a	$7.0 \pm 0.28a$	6.5 ± 0.14^a	7.0 ± 0.27^a
	33	6.8 ± 0.37^a	5.8 ± 0.19^b	5.7 ± 0.47^a	5.8 ± 0.51^b
	36	6.2 ± 0.14^b	5.5 ± 0.42^b	5.3 ± 0.14^b	5.5 ± 0.42^b
Free CO_2 (mg/L)	30	6.0 ± 0.07^a	7.0 ± 0.07^a	6.0 ± 0.07^a	7.0 ± 0.14^a
	33	8.0 ± 0.14^b	8.0 ± 0.04^b	9.0 ± 0.07^b	9.0 ± 0.07^b
	36	8.0 ± 0.12^b	9.0 ± 0.07^b	11.0 ± 0.21^b	10.0 ± 0.07^b
pH	30	8.60 ± 0.28	8.83 ± 0.15	7.25 ± 0.07	7.15 ± 0.07
	33	8.60 ± 0.0	8.66 ± 0.11	7.55 ± 0.21	6.90 ± 0.14
	36	8.40 ± 0.70	8.50 ± 0.10	7.35 ± 0.07	7.20 ± 0.14
Total alkalinity (mg/L)	30	130.0 ± 9.1	135.0 ± 7.1	108.0 ± 8.1	114.0 ± 9.6
	33	115.0 ± 7.2	95.0 ± 8.7	122.0 ± 7.2	112.0 ± 8.3
	36	115.0 ± 8.3	115.0 ± 7.1	115.0 ± 7.8	130.0 ± 9.7

Values of a single water quality parameter in a column with different alphabetical superscripts are significantly ($p < 0.05$) different.

Blood glucose is one of the potential indicators to assess stress in fish to any adverse environmental conditions (Silbergeld, 1971; Beyea et al., 2005). In the present study, the increase of glucose levels resulted from the conversion of glycogen into glucose to meet the extra demand of metabolic energy under stressed conditions caused by high temperature. The increased level of glucose is required to meet the new energy demands (gluconeogenesis) of stressed fish (Winkaler et al., 2007). For instance, two stress hormones, namely glucocorticoids and catecholamine cause increased glucose levels beyond the normal at stressed condition and have been shown to cause hyperglycemia in fish (Pickering, 1981; Banaee et al., 2011). Moreover, thermal stress may negatively affect the functions of major organs of fish including the liver and kidney affecting the homeostasis of fish (Sharmin et al., 2015). Consistent with the previous works, the present study observed the response of Indian major carp, rohu exposed to high temperature induced metabolic stress causing significant increase in plasma glucose concentration.

Temperature can cause stress because oxygen solubility decreases in water with increasing temperature (Cech and Brauner, 2011). It has been reported that at raised temperature (36 °C), fish not only deal with thermal stress but also confront hypoxia (Carvalho and Fernandes, 2006; Hedayati and Tarkhani, 2014). In the present study, dissolved oxygen (mg/L) values were decreased significantly ($p < 0.05$) with increasing temperature. However as the aerators were used, dissolved oxygen level in the rearing tanks was not hypoxic for the experimental fish. Therefore, stress associated affects in the present study were not caused by hypoxia, but undoubtedly caused by raised temperature alone.

Among all of the blood parameters monitored in the present study, the increasing number of WBC and neutrophils and decreasing number of lymphocytes in the blood of rohu were clearly evident throughout the study period. We believe that these parameters are the most useful to verify, if rohu exposed to high temperature are stressed or not. Rohu is mainly distributed and commonly farmed in a few countries of South Asia in the tropics, where, in the summer months, water temperature is generally very high. Even higher temperatures are predicted in the coming years due to global climate change. This would definitely cause seasonal changes of the hemato-biochemical indices with impact on the feeding, growth, production, survival and general wellbeing of rohu and other commercially important aquaculture species.

5. Conclusion

We studied the effects of high temperature on hemato-biochemical parameters and erythrocytes cellular and nuclear structures in rohu. Chronic exposure to high temperature decreased Hb and RBCs and increased the WBC and blood glucose levels of the fish. Frequencies of ECA and ENA were found to be increased at high temperature. High temperature significantly increased the number of neutrophils whilst decreasing the number of lymphocytes. Overall, this study confirmed that exposure to high temperature is stressful to Indian major carp rohu. This is particularly important given the predicted changes in water temperature due to global climate change.

Acknowledgements

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jtherbio.2019.102450.

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