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Effect of thermal stress of short duration on the red blood cell parameters of *Barbus balcanicus* Kotlik, Tsigenopulos, Rab, Berrebi, 2002

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In this study, red blood cell parameters of *Barbus balcanicus* Kotlik, Tsigenopulos, Rab, Berrebi, 2002 subjected to acute thermal stress were investigated. Fish were caught by electro fishing in the Suturlija river, a small tributary of the river Vrbas (N latitude 44˚44' 38˝, E longitude 17˚ 09' 10˝) in summer (July) and transported to the laboratory. Fish were randomly distributed in four aquaria of 30 L each. In all the four aquaria, the water temperature was continuously kept at 19°C by appropriate devices. After period of adaptation (three weeks), half of the fish (24) were used as a control group while the other 24 were subjected to thermal stress by raising the water temperature to 29°C (10°C increase) in 60 min. In both control and thermally treated, fish blood was collected by heart puncture according to "Animal welfare act". Analyses were performed with native blood, without addition of any anticoagulant and the values of red blood cell count (RBC), haemoglobin concentration, packed cell volume (PCV), Corpuscular Volume (MCV), Mean Corpuscular Haemoglobin (MCH) and Mean Corpuscular Haemoglobin Concentration (MCHC) were determined. In the thermally treated fish, the values of PCV and MCV increased ($p = 0.0003$ and $p = 0.0147$ respectively), and those of MCHC decreased substantially ($p = 0.0001$).

Key words: Haematology, temperature, *Barbus balcanicus*.

INTRODUCTION

Over the last decades, the research in fish reaction to high sublethal temperatures has been increasingly important. This is correlated with an average temperature increase tendency and other climate changes in many regions of the world (Hari et al., 2006). In Europe it is manifested primarily through the increase in the number of warmer winters and specific summer periods which are characterised by changing short term periods of above average high temperatures with periods of heavy precipitations and high humidity during summer. Therefore, the interest in temperature influence research as one of the basic ecological factors is rising, especially for the effect of additional heating that surface layers and small freshwater bodies are exposed to. It is common for many water bodies (both natural and man-made) to experience daily fluctuations of >4°C during summer, and some may fluctuate as much as 10°C and thus cause thermal stress (Bevelhimer and Bennett, 2000). Although the amount of daily fluctuations in air temperature is probably the most important factor in dictating the water

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temperature fluctuation, other factors, such as the size of the water body, volume flow rate, degree of mixing and exposure to solar radiation are also important. Rapid and considerable changes in ambient temperature inevitably cause stress in animals and modify physiological conditions of their organism resulting in the "general adaptation syndrome", as defined by Selye (Selye, 1950; Iwama et al., 2004). The course and outcome of stress depend mostly on the intensity and duration of thermal regime changes.

Due to the ectothermic nature of teleost fish, the surrounding environment dictates the body temperature and fish become vulnerable to unseasonal thermal changes that may occur. Acute temperature increases have been found to initiate primary as well as secondary stress response, both of which produce osmoregulatory and metabolic effects in many species of fish (Ryan 1995; Ackerman et al., 2000; Basu et al., 2001; Mesa et al., 2002; Gollock, 2002).

Fish can adapt to the increased temperature of its habitat to a certain extent, but when a particular temperature exceeds the threshold value, thermal stress occurs. The temperature at which stress begins to occur is largely dependent on a fish's prior experience or acclimation. Even with acclimation, there is temperature beyond which stress occurs regardless of previous acclimation at sub-stressful temperatures (Barton, 2002).

In view of insufficient knowledge of thermal stress in fish, numerous experiments were conducted with the aim to contribute to better understanding of physiological conditions of the fish organism after thermal stress (Fries, 1986; Bevelhimer and Bennett, 2000).

The influence and state of thermal stress are difficult to monitor exclusively under natural conditions in which an array of factors are changing; therefore, such research can be best conducted under controlled laboratory conditions (Šimić and Ivanc, 1999).

The greatest part of the previously conducted research in fish stress was primarily focused on the description of the neuroendocrine aspect of stress state and neuroendocrine response in different species. This traditional approach has not been changed over the last twenty years (Iwama et al., 2004). There is a need for a new approach to fish stress research so as to gain better understanding of this physiological phenomenon. Thereby a vital precondition for such research is knowledge of physiological range of variation of a studied parameters (normal values) and environmental conditions in which research is conducted.

Various studies have shown that haematological parameters can provide satisfactory information on physiological response of fish to environmental changes because blood constituents respond rapidly to any factor affecting homeostasis, including the influences of the habitat. Besides that, fish blood is easy to collect (Cazenave et al., 2005; Lohner et al., 2001; Elahee and Bhagwant, 2007).

Fish blood parameters, such as the size and number of erythrocytes and the concentration of haemoglobin, vary dependent on ambient conditions (Andersen et al., 1985; McClanahan et al., 1986; Allen, 1993; Hlavova, 1993; Ytrestoyl et al., 2001), metabolic demands (Pickering, 1986; Bollard et al., 1993; Takeda, 1993) and taxonomic differences (Atkinson and Judd, 1978; Van Vuren and Hattingh, 1978).

Since erythrocyte characteristics determine significantly the efficiency of oxygen transport from the respiratory system to tissues (Holland and Forster, 1966; Nikinmaa and Salama, 1998), changes in their number and volume could influence metabolic performance (Atkinson and Judd, 1978; Beitinger et al., 1985; Bollard et al., 1993; Hlavova, 1993).

As opposed to terrestrial animals, fish particularly face a very variable environment and emphasized alterations in oxygen availability. However, a variety of physiological adjustments are observed in fish exposed to environmental and/or tissue hypoxias in order to improve the O_2 transfer (de Souza and Bonilla-Rodriguez, 2007).

When the environmental temperature increases, the concentration of water oxygen falls, but an increase in metabolic rate, and therefore in tissue oxygen demand, occurs. As is also the case for other ectothermic vertebrates, fish can adapt to changes in environmental temperature and hypoxia by increasing their total haemoglobin content (Brix et al., 2004).

The objective of this paper was to study the influence of an acute increase of temperature and metabolic rate on basic blood parameters as oxygen transport system.

The effect of thermal stress was studied on the *Barbus balcanicus,* a species inhabiting smaller water bodies often exposed to temperature fluctuatiation. During the experiment, the fish were subjected to substantial temperature changes, similar to the ones in their natural habitat, especially during summer.

MATERIALS AND METHODS

Specimens of *Barbus balcanicus* (48 individuals) used in this study inhabited the Suturlija river, a small tributary of the river Vrbas, Bosnia and Herzegovina (N latitude 44˚ 44' 38˝, E longitude 17˚ 09' 10˝). This stream was chosen because it is characterised by significant changes of volume flow rate and water temperature. The fish were captured by electrofishing - using a pulse direct current unit IG 600 (peak power output 1.2 kW) during summer (July).

The fish caught were placed in containers filled with a sufficient quantity of water and equipped with aerators. They were then transported to the aquarium of the Faculty of Natural Sciences and Mathematics, University of Banja Luka. After that, by random sampling, the fish were distributed in four aquaria, each 30 L and supplied with the water from the Suturlija. The fish were let to acclimate to the laboratory conditions for three weeks before the experiment started. That period also allowed recovery from electrofishing, transportation and handling (Gollock, 2002).

The overall mean body mass of fish used for the experiment was 27.33 g and the total body length was 14.12 cm. Twelve (12) fish were placed in each aquarium so that fish mass per litre of water was 11 g.

Table 1. Mean values of water temperature, oxygen concentration and saturation rate in the control aquaria at 19 °C (1 and 3) and in the aquaria in which the temperature was increased from 19 to 29°C (2 and 4).

Aquarium	Temperature (°C)	Dissolved oxygen (mg/l)	Oxygen saturation rate (%)
1	19	8.31	92.23
2	29	6.58	86.13
3	19	8.62	95.67
4	29	6.69	87.56

The Fulton coefficient was calculated as follows:

$F = bm/l^3 \times 100$

Where, bm = body mass and l = standard body length.

In all the four aquaria, the water was conditioned with appropriate devices and the temperature was kept at 19°C. Besides, half of the water volume in each aquarium was replaced each day with the water brought from the native stream.

The water quality was assessed daily by testing the dissolved oxygen concentration, pH, COD and ammonia concentration.

After the period of adaptation, half of the fish (24) were used as a control group while the other 24 were subjected to thermal stress. The treatment was conducted according to European directive 2010/63/EU and the “Animal welfare act” (“Official Gazette of the Republic of Srpska”, No. 111/2008).

24 individuals from the two aquaria were exposed; 12 at a time, to thermal stress of short duration by raising the water temperature by 10°C in a 60 min period.

To achieve a rapid increase in the water temperature, maximal heating by the circulating heater unit was applied. The temperature rose from the initial 19°C (±1°C) to 29°C after 1 h, before it became steady and was kept so for 20 min.

Each experiment started at the same time to minimise the effect of diurnal variations on physiological parameters (Suski et al., 2007).

Blood sampling

In both the control and thermally treated fish, blood was collected in the morning by heart puncture with a sharp and wide sterile needle (1.0 to 1.2 mm). The analyses were performed with native blood, without any anticoagulant added.

Haematological analyses

The haematological status of fish was determined on the basis of haemoglobin concentration (Hb), packed cell volume (PCV), number of erythrocytes (red blood cell count – RBC), Mean Corpuscular Volume (MCV), Mean Corpuscular Haemoglobin (MCH) and mean corpuscular haemoglobin concentration (MCHC).

Haemoglobin concentration was determined spectrophotometrically using an assay kit (Sigma 527-A).

PCV was measured immediately after blood sampling, in duplicate, in pre-heparinised micro-haematocrit tubes, centrifuged at 12250 x *g* for 5 min (Hettich, Universal 32 haematocrit 24), using a haematocrit reader.

Counting of erythrocytes was performed in a haemocytometer after diluting the blood, according to Kekić and Ivanc (1982).

Haematological indices were calculated using the values for haemoglobin concentration, PCV and RBC.

$$MCV = \frac{\mathrm{PCV}}{RBC/l}$$

$$MCH = \frac{\mathrm{Hb}/\mathrm{l}}{\mathrm{RBC}/\mathrm{l}}$$

$$MCHC = \frac{\mathrm{Hb}/\mathrm{l}}{\mathrm{PCV}}$$

Body mass and morphometric characteristics

Body mass, standard and total body length were determined 1 h after the blood was collected.

Statistical analysis

Statistical analyses were done using Microsoft Excel and SPSS 11.5. Normality of distribution was tested by Shapiro Wilk test, while the comparison was done using post hock LSD test (ANOVA). Also, confidence interval of 95% was listed. Level of statistical relevance was 0.05.

RESULTS

Oxygen regime

The values of oxygen concentration and oxygen saturation rates are shown in Table 1. The values of saturation were above 80% in all the used aquaria, which indicate that they were within the normal range for that species.

Body mass and morphometric characteristics

Morphometric characteristics were analysed in 24 individuals that had not been subjected to thermal stress but kept under the same laboratory conditions and served as the control group of individuals. In this group, the mean value of the total length of individuals amounted to 14.02 cm, the mean value of the standard length was 11.75 cm, and the mean mass amounted to 28.07 g (Table 2).

24 individuals of *B. balcanicus* with the total mass of 667.00 g were subjected to thermal stress. Their mean total length amounted to 14.23 cm, the standard length was 12.03 cm, while the mean body mass amounted to 26.71 g (Table 2). The fish body mass was 11.13 g/l

Table 2. Total length, standard length, mass and Fulton coefficient of control and thermally treated *B. balcanicus*.

Statistic	Total length (cm)	Standard length (cm)	Mass (g)	Fulton coefficient
Control fish				
Mean	14.02	11.75	28.07	1.56[a]
Standard deviation	2.45	2.18	20.75	0.15
Minimum	9.80	8.00	7.20	1.35
Maximum	22.40	19.20	112.31	1.98
95% Confidence interval for mean				
Lower bound	13.04	10.86	19.74	1.50
Upper bound	15.02	12.63	36.17	1.62
Coefficient of variation (%)	17.47	18.55	73.92	9.44
Thermally treated fish				
Mean	14.23	12.03	26.71	1.46[b]
Standard deviation	1.76	1.53	13.31	0.14
Minimum	11.70	9.90	12.10	1.24
Maximum	21.10	17.90	83.80	1.79
95% Confidence interval for mean				
Lower bound	13.50	11.40	21.22	1.40
Upper bound	14.96	12.66	32.21	1.52
Coefficient of variation (%)	12.37	12.72	49.83	9.73

Mean values of the same parameter with different letters in superscript were significantly different ($p \leq 0.05$).

water.

The thermally treated fish had a slightly higher mean value of total and standard lengths and a lower mean body mass value. However, the differences were not statistically significant.

The thermal treatment resulted in a significant decrease in the Fulton coefficient (0.019). From the Fulton coefficient equation, it is evident that even a slight change in body mass (with almost constant length) changes the Fulton coefficient to a great extent.

Haematological parameters

Haematology of *B. balcanicus* exposed to thermal stress was characterised by: (a) the increase in MCV with a resulting increase in PCV and (b) a decrease in haemoglobin concentration in a litre of erythrocytes (MCHC).

The obtained results of the analysed haematological parameters showed that there were statistically significant differences between the control and the thermally treated fish (Table 3).

Temperature effects were observed in most of the monitored haematological parameters.

In the thermally treated individuals, a significant increase in values of PCV ($p = 0.0003$) and MCV ($p = 0.0147$) was recorded. On the contrary, MCHC values (0.0001) decreased significantly.

In those fish slightly higher values of RBC and haemoglobin concentration were also found. The control individuals had higher MCH values, but the difference was not statistically significant.

Decrease in MCHC values can be related to increase in erythrocyte volume (MCV) which is not followed with adequate increase of haemoglobin in them (MCH). However, slight increase of haemoglobin concentration indicates the beginning of its synthesis in mature erythrocytes.

As a consequence of the aforementioned changes, the distributions of the values of the analyzed haematological parameters were also influenced by the thermal treatment. According to Shapiro-Wilk test, the distribution of MCH and MCHC values changed significantly after the treatment. Moreover, they were out of normal range (0.035 and 0.036 respectively) and became positively skewed (1.235 and 1.359 respectively) with majority of the data concentrated on the left of the curve and a few higher values on the right. This appearance of the higher values of Hb in individual red blood cells indicates the beginning of its synthesis in them.

Furthermore, in those fish, the distribution of RBC,

Table 3. Haematological values of control and thermally treated *B. balcanicus.*

Statistic	Hb (g/l)	PCV (l/l)	RBC ($x10^{12}$/l)	MCV (fl)	MCH (pg)	MCHC (g Hb/l eryt.)
Control fish						
Mean	70.52	0.320[a]	1.150	284.56[a]	61.98	219.73[a]
Standard deviation	10.77	0.056	0.155	47.81	10.35	23.39
Minimum	44.40	0.200	0.780	201.90	47.51	163.40
Maximum	90.70	0.500	1.900	378.40	83.14	285.90
95% confidence interval for mean						
Lower bound	65.97	0.301	1.084	262.30	57.61	207.74
Upper bound	75.07	0.348	1.215	303.08	66.35	231.72
Coefficient of variation (%)	15.27	17.56	13.50	16.80	16.69	10.65
Thermally treated fish						
Mean	71.32	0.390[b]	1.210	323.78[b]	59.42	186.05[b]
Standard deviation	7.63	0.056	0.122	57.33	8.84	26.63
Minimum	59.30	0.200	0.960	215.80	47.00	144.90
Maximum	87.03	0.500	1.540	492.70	86.80	271.00
95% confidence interval for mean						
Lower Bound	68.02	0.365	1.159	299.01	55.60	174.54
Upper Bound	74.63	0.413	1.264	348.58	63.25	197.57
Coefficient of variation (%)	10.70	14.31	10.05	17.71	14.88	14.32

Mean values of the same parameter with different letters in superscript are significantly different ($p \leq 0.05$).

PCV, MCH and MCHC values was leptokurtic (1.716, 2.367, 2.828 and 3.656 respectively), indicating heterogeneity and infrequent extreme individual values.

DISCUSSION

Influence of thermal stress on energy expenditure

Thermal stress of *B. balcanicus* resulted in decrease of Fulton coefficient which depends on the values of standard body length and body mass. It is in accordance with the fact that during the metabolic rate increase caused by temperature elevation (Smith, 1989; McGoogan and Gatlin**,** 1998; Clarke and Johnston 1999; Campbell et al., 2008; Otto and Zahn, 2008), fish satisfy their higher energy demand by consumption of fat and as a consequence of that, reduction in body mass occurs.

Also, Schreckenbach (2002) found that in the common carp, the exposure to temperature increase from 3 to 20°C in the course of 3 h, and was followed by a higher metabolism rate during the following 14 days and increased consumption of the half of its body fat reserves.

Influence of thermal stress on haematological parameters

The increase in the volume of MCV in *B. balcanicus* exposed to increase of water temperature is probably caused by swelling of erythrocytes under adrenergic influence (Jensen, 2004), which is typical for the alarm phase of stress reaction.

According to Schindler and de Vries (1986) and Speckner et al. (1989) in such mature circulating erythrocytes haemoglobin synthesis begins. In the same time, increase of haemoglobin affinity to oxygen occurs caused by changes of pH to neutral or slightly alkaline.

In this study, the increase of PCV was found in thermally treated fish what is consistent with the research of Barton (2002) that PCV is a measure of the cellular fraction of blood and is a common stress indicator for fish.

Also, Ruane et al. (2001) concluded that if there is no increase in PCV values, there is no need for extra oxygen in the fish organism. Based on that, changes of PCV values can be used as reliable criterion for determination of organism oxygen demand.

In one of our earlier studies, a significant increase of PCV was established in *Carassius gibelio* after it had been exposed to the ambient temperature elevation of 10°C (Dekić et al., 2011).

Similar to the results of this study, the effect of thermal stress on various blood parameters was noticed in the studies of *Dicentrachus labrax* (Kacem et al., 1987), exposed to a rapid increase of temperature within 1 h.

Reaction of different fish species to temperature increase

Different fish species show different changes in their haematology after exposure to increase of water temperature. Gollock et al. (2005) obtained very interesting results. They showed that there is a significant increase in both PCV and haemoglobin concentration levels in eels after 6 h exposure to elevated temperatures. On the other hand, there were no significant changes in MCHC. The parallel increases in PCV and haemoglobin concentration in the absence of significant changes in MCHC suggest an increase in the number of circulating erythrocytes without significant erythrocytic swelling.

Perry and Reid (1992) also contributed to this phenomenom. They found that catecholamines induce minimal erythrocyte swelling in anguillids, as opposed to their significant effect and resultant reduction in MCHC in other species (Nikinmaa and Salama, 1998).

Similar to our present research, Smit et al. (1981) conducted an experiment on the following three fish species: tilapia (*Sarotherodon mossambicus*), common carp (*Cyprinus carpio*) and rainbow trout (*Oncorhynchus mykiss*). The water temperature in the laboratory was approximately 19°C. After two months of acclimation, the individuals of each species were adapted to three different temperatures (15°C, 20°C and 25°C) for three weeks and then haematological parameters were analysed.

In the tilapia exposed to the increased temperature, the mean value of RBC also increased, while in the common carp and trout, these values remained constant. The increased number of erythrocytes was not accompanied by increased haemoglobin concentration.

In all the species, MCV was not related to temperature. In the carp, both MCH and MCHC did not change with respect to temperature and in the trout MCH remained unchanged.

MCH and MCHC did not show temperature dependence in the tilapia either, while in the trout, a positive relationship was determined only with respect to MCHC.

Finally, it may be concluded that in various species, the response of haematological parameters to the increased temperature is different and depends on the ability of adaptation of individual species and on the length and intensity of the change in temperature.

Speckner et al. (1989) found that density to volume relationship differs greatly between the common carp and human erythrocytes. In other words, mammalian red cells shrink in volume during ageing, but retain constant haemoglobin content. Thus, the increase in haemoglobin concentration, and therefore in density, is caused by the reduction in volume. On the other hand, during ageing, carp red cells increase not only with respect to haemoglobin content, but also to volume. A greater increase in the haemoglobin content than in the volume of erythrocytes causes the increase in haemoglobin concentration and density. Erythrocytes can only increase their haemoglobin content by synthesis. From the continuous rise in the haemoglobin content, it is obvious that fish erythrocytes must synthesis haemoglobin after being released into the circulation. Incubations with [2-14C] glycine provided information about haemoglobin synthesis *in vitro.* This amino acid marker can label the globin component intra-translationally and is utilised in haem synthesis (Speckner et al., 1989).

Similar to this study, Houston and Murad (1992) found in goldfish, *Carassius auratus* (Linnaeus, 1758) exposed to abrupt heat shock (15 to 25°C), the changes in peripheral erythrocyte populations in terms of red cell numbers, haemoglobin concentration, PCV, MCV and MCH.

General hematological responce in stress

Haematologic parameters represent not only reliable stress indicators but also indicators of the state of fish organism and different influences of their environment (Rowan, 2007).

Acerete et al. (2004) studied the most important physiological blood parameters of primary and secondary stress responses of the perch subjected to stress by transport and acute handling. They found an increase in PCV values and in the number of red blood cells.

Stress in fish cause changes in different physiological parameters. Thus, Beyea et al. (2005) found out that in *Acipenser brevirostrum*, there was a significant interaction between ploidy and post-stress time for blood haemoglobin concentrations with diploids demonstrating elevated haemoglobin content 6 h after experiencing stress.

In *Albula vulpes,* Murchie et al. (2009) found out that stress caused by relocation process evoked secondary stress responses at the metabolic, osmoregulatory and haematological levels as indicated by changes in blood glucose, lactate, PCV and ion values, with respect to control fish.

Suski et al. (2006) found out that after 4 min of exercise, *Albula vulpes* exhibited physiological and biochemical changes that included significant increases in PCV with respect to resting values.

Conclusions

In *B. balcanicus* exposed to thermal stress due to temperature elevation of 10°C in a 60-minute period the mean PCV value increased substantially. The mean MCV value was also significantly higher in thermally treated individuals indicating that the average erythrocyte volume had increased. MCHC values in thermally treated individuals were significantly lower, indicating that the average quantity of haemoglobin in one litre of erythrocytes decreased in this group of individuals. At the same time, some parameters also changed in fish exposed to the temperature increase, but not significantly. RBC increased, MCH value decreased while haemoglobin concentration remained unchanged or increased slightly.

The changes in parameters are the result of the response of the organism to the changed circumstances in their environment, that is their specific response in the form of "general adaptation syndrome" – stress which improved oxygen transport mechanism.

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