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EFFECTS OF THERMAL STRESS ON HEMATOLOGICAL AND METABOLIC PROFILES IN BROWN BULLHEAD, *Ameiurus nebulosus* (LESUEUR, 1819)

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Abstract

Physiological stress response of Brown bullhead (Ameiurus nebulosus) was studied under laboratory conditions. This species is well known for the resistance and adaptability to environmental factors. In this sense, metabolic and hematologic profiles were determined for low (6^o C), medium (18^o C) and high (31^o C) water temperatures. An increase in blood cell numbers (RBC, WBC) and hematocrit (Ht) was determined under high temperature conditions, as a response to low dissolved oxygen. Platelet count (PLT) remained relatively constant regardless of the water temperature. The metabolic profiles showed significant and very significant differences for most of the analyzed indices. The differences were due to variations in temperature that influenced appetite and ingestion of feed, respectivelly the respiratory function by vasoconstriction or hypoxia.

Key words: *Ictaluridae, invasive species, blood analysis, medial parameters, adaptability.*

INTRODUCTION

Brown bullhead (*Ameiurus nebulosus*) is part of the *Ictaluridae* family. It is native to North America and has its origin area between Hudson Bay, St. Lawrence - Great Lakes, the American Atlantic limit and the Mississippi River (Craig et al., 2015). It was introduced in various parts of Europe (Holčik, 1991), Asia and the Pacific Islands, including New Zealand (Collier et al., 2016) at the end of the XIX^{-th} century (1885). The data regarding the occurrence of this species in Romania is controversial. Vasiliu (1959) mentioned its artificial introduction at the beginning of the XX^{-th} century (1908). Bănărescu (1964) mentioned its migration from western European countries and noticed its presence in the Romanian Danube sector, Baziaş area, around 1940. It was introduced for diversity purposes like sport fishing in these new habitats. It also became the subject of aquaculture (Dunham, 2006; Marković et al., 2012) and artificial reproduction (Fobes, 2013).

The Brown bullhead is recognized for its adaptability to difficult environmental conditions (Kapusta et al., 2010; Popescu, 2014), so it can withstand low dissolved oxygen values, low water pH (Frank, 2015) and large temperature variations (Scott and Crossman, 1973). All these physiological features correlated with specific anatomical structures (well-developed olfactory and gustative analyzers, venomous glands, robust body formations) (Moldowan et al., 2015; Popescu et al., 2015) contribute to a very good adaptation in the new habitats, becoming a dominant species with major influences on endemic fish (Lenhardt et al., 2010). Brown bullhead is usually found in benthic zones, were it competes for food and resources with various demersal species (Kapusta et al., 2010). When found in natural water bodies, a high survival rate is noticed because of its territorial and parental-protective behaviour. The feeding spectrum is represented by chironomidae larvae (Kline and Wood, 2011; Frank, 2015), diptera, fish eggs, fish juveniles and crustaceans such as crayfish (Keast, 1985; Raney and Webster, 2011). All the above are reasons for which the Brown bullhead is considered a harmful species. Measures are

currently taken to limit its spread in both artificial and natural environments (Danalache et al., 2017).

In the case of aquatic organisms, temperature along with other physical and chemical factors play an essential role in the progress of physiological processes expressed by growth and intensity of metabolism (Peck et al., 2005). Each species has optimal temperature range, in which these physiological processes run properly. If these conditions are not provided, a stressful state appears and has corresponding consequences: decrease in metabolic rate, slow growth rate and installation of pathologic state (Barton, 2002).

Blood is the most efficient stress indicator (Hattingh, 1977; Marin et al., 2015; Simide et al., 2016). Biochemical and hematological studies on fish are usually made for high economical value species. In general, Brown bullhead studies on hematological and biochemical properties were sporadic and made the subject of cytological and pathophysiological researches (Rowan, 2007; Baumann et al., 2008). Despite the fact that the Brown bullhead is considered an invasive and undesirable species in Romania, studies regarding its physiological traits have not been carried out. Our study shows the expression of thermal stress in the blood constituents of the Brown bullhead. The obtained results give fundamental arguments in understanding the process of adaptation by physiological mechanisms tested at extreme temperatures.

MATERIALS AND METHODS

The Brown bullhead specimens from this study were sampled from Stejeriș Lake, Cluj County. Three groups of 50 specimens were constituted. The average body weight (BW) was 88.64±2.18 g and the average total length (TL) was 19.85±0.14 cm. Each group was placed in 500L water tanks for 21 days at three different temperatures [Group 1: 6^{o}C - low temperature (LT); Group 2: 18^{o}C - normal temperature (NT); Group 3: 31^{o}C - high temperature (HT)].

In order to observe the physiological status of Brown bullhead and the influence of thermal stress on the hematological profile, blood samples were collected by caudal vein puncture from 10 randomly selected specimens from each group. The samples were transferred into 95 IU (4 mL) Li-Heparin anticoagulant vacutainers and transported under refrigeration conditions (+ 2°C) to the hematology laboratory of UASVM Cluj-Napoca. The following determinations were performed: HGB (hemoglobin), HCT (hematocrit), MCV (mean corpuscular volume), MCH (mean corpuscular hemoglobin), MCHC (mean corpuscular hemoglobin concentration), RBC (red blood cell count) WBC (white blood cell count), PLT (platelets) and the leukocyte formula. The hematocrit was determined by centrifugation at 12.000 rpm/3 min. Hemoglobin concentration and erythrocytes count were determined by spectrophotometry techniques (UV-VIS Screen Master Touch). End-Point colorimetric reaction was used for hemoglobin determination. Readings were performed in the NIS range spectra using as reagent 0.4% ammonium hydroxide NH_4OH in distilled water. Turbidimetric and colorimetric EP-type reaction with visible reading was used for the determination of erythrocytes using as a reagent acetic solution of sodium sulfate Na_2SO_4 in distilled water (Gowen's reagent). The leukocyte formula was obtained by microscopic reading of the obtained blood smears. Coloration was performed by Reag-Quick-Panoptic [Reag-Fix Panoptic (fixative) - Reag-Red Panoptic (eosinophil) - Reag-Blue Panoptic (basophil)] and cleaning with bidistilled water. RBC (MCV, MCH, and MCHC) was calculated based on the formulas reported by Ghergariu et al. (1999):

Mean Corpuscular Volume

$$MCV = \frac{HT \times 10}{RBC}$$

Mean Corpuscular Hemoglobin

$$MCH = \frac{HGB \times 10}{RBC}$$

Mean Corpuscular Hemoglobin Concentration

$$MCHC = \frac{HB \times 100}{HCT}$$

The biochemical profile obtained by spectrophotometry techniques with (λ) wavelength reading includes: protein profiles [TP -total protein (λ = 546 nm) (EP); ALB - albumin (λ = 630 nm) (VIS); GLOB -globulin (λ = 405 nm) (EP); BUN -blood urea nitrogen

(λ = 340 nm) (UV); CREAT-creatine (λ = 510 nm) (NIS)], lipid profile [CHOL-cholesterol (λ = 500 nm) (NIS); TGC-triglyceride (λ = 550 nm) (EP)], carbohydrate profile [BG-blood glucose (λ = 50 nm) (NIS)], mineral profile [TC-total calcium (λ = 575 nm) (EP); P -phosphorus (λ = 340 nm) (UV); Fe-blood iron (λ = 630 nm) (EP); Na -sodium (λ = 405 nm) (NIS); K -potassium (λ = 380 nm) (UV)], enzyme profile [ALP-alkaline phosphatase (λ = 405 nm) (CR); GGT-gamma-glutamyl transpeptidase; AST -aspartate aminotransferase; ALT -alanine aminotransferase; AM EP-serum amylase; LDH-lactate dehydrogenase (λ = 340 nm) (CR); LIP -lipase (λ = 580 nm) (CR); CPK -creatine phosphokinase (λ = 340 nm) (CR); TB -total bilirubin]. Oxidative stress was also determined by the antioxidant SOD-superoxide dismutase enzyme (λ = 505 nm). The statistical interpretation of the obtained data from biochemical analyzes was performed with the GraphPad Prism 6 software, using ANOVA test for multiple comparisons. Tables and charts were designed in Word and Excel, Microsoft Office 2010.

RESULTS AND DISCUSSIONS

Hematological analyzes show a numerical increase in blood figurative elements, proportionally to the increase of temperature. For leukocytes, at 6°C the mean value was 16.35 ± 1.596 WBC x 10^3/μL, at 18°C the mean value was 17.20 ± 0.536 WBC x 10^3/μL (d = 0.85**, P <0.01) and at 31°C the mean value was 18.12 ± 0.529 x 10^3/μL.

The lowest mean value for erythrocytes was obtained at 18°C (RBC = 0.39 ± 0.016 x 10^6/μL) and the maximum mean value was obtained at 31°C (RBC = 0.88 ± 0.11 x 10^6/μL). Intermediate mean value was obtained at 6°C (RBC = 0.50 ± 0.032 x 10^6/μL).

Correlations between blood cells counts (WBC and RBC) and temperature were mentioned by Bozorgnia et al. (2011) and Witeska (2013). The increase of figurative blood count is a stress state indicator.

The physiological response of fish regarding high water temperatures (when dissolved oxygen level is low) is expressed by the increase of erythrocyte counts. In response to environmental conditions, fish specific hematopoiesis in the kidneys and spleen (Maekawa and Kato, 2015) is accelerated and a higher number of erythrocytes are able to carry more efficiently the reduced amount of dissolved oxygen in the water.

The hemoglobin (Hg) varies from 5.5±0.196 g/dL (at 18°C), to 7.66±0.638 g/dL (at 31°C) and 10.2±0.388 g/dL (at 6 °C). Fish have specific anatomical and physiological adaptations that coordinate and adjust the amount of hemoglobin in the blood flow. This mechanism is based on the variation of medial conditions and the chemical modulation stimulus. Both act on the oxygen requirements of different tissues (de Souza and Bonilla-Rodriguez, 2007). Temperature and dissolved oxygen levels from water are usually found in inverse relationship. Tissular oxygen demand is no longer provided under hyperthermia conditions. The physiological response to this thermal stress factor is manifested by variations of cortisol and blood glucose (Martínez-Porchas et al., 2009) witch lead to blood pH alterations. Hemoglobin is highly sensitive to pH changes, Bohr reduction of Hg-O_2 affinity) and Root (decrease in carrying capacity) effects occur under stress conditions (Rummer and Brauner, 2015). In Table 1 high levels of blood glucose (BG) are presented, both hypo- and hyperthermia leading to increased hemoglobin when thermal stress is installed.

The results of our study indicate that the highest hemoglobin concentration occurs for low water temperatures (LT). Collateral and cumulative physiological adaptations may be the response to environmental factors as previously described. Increased hemoglobin levels occur in hypothermia because of vasoconstriction (especially in skeletal muscle capillaries) correlated with significant increase in mitochondrial density (Johnson and Dunn, 1987). Blood circulation is carried out at a low rate, especially at the peripheral level and the demand for tissular oxygen is no longer assured. A higher concentration of hemoglobin is necessary in this case.

By comparing our hypothermic stress generating experiment to the conditions of the Arctic environment, some adaptive differences can be

observed. Fish from Arctic regions have low hemoglobin levels (directly correlated with myoglobin) (Wells et al., 1980). This is a phylogenetic adaptability of fish species living in cold waters, which have a high level of nitric oxide (NO) that acts as a vasodilator (Sidell and O'Brien, 2006). Brown bullhead is not native to cold areas, thus lacking this phylogenetic adaptation.

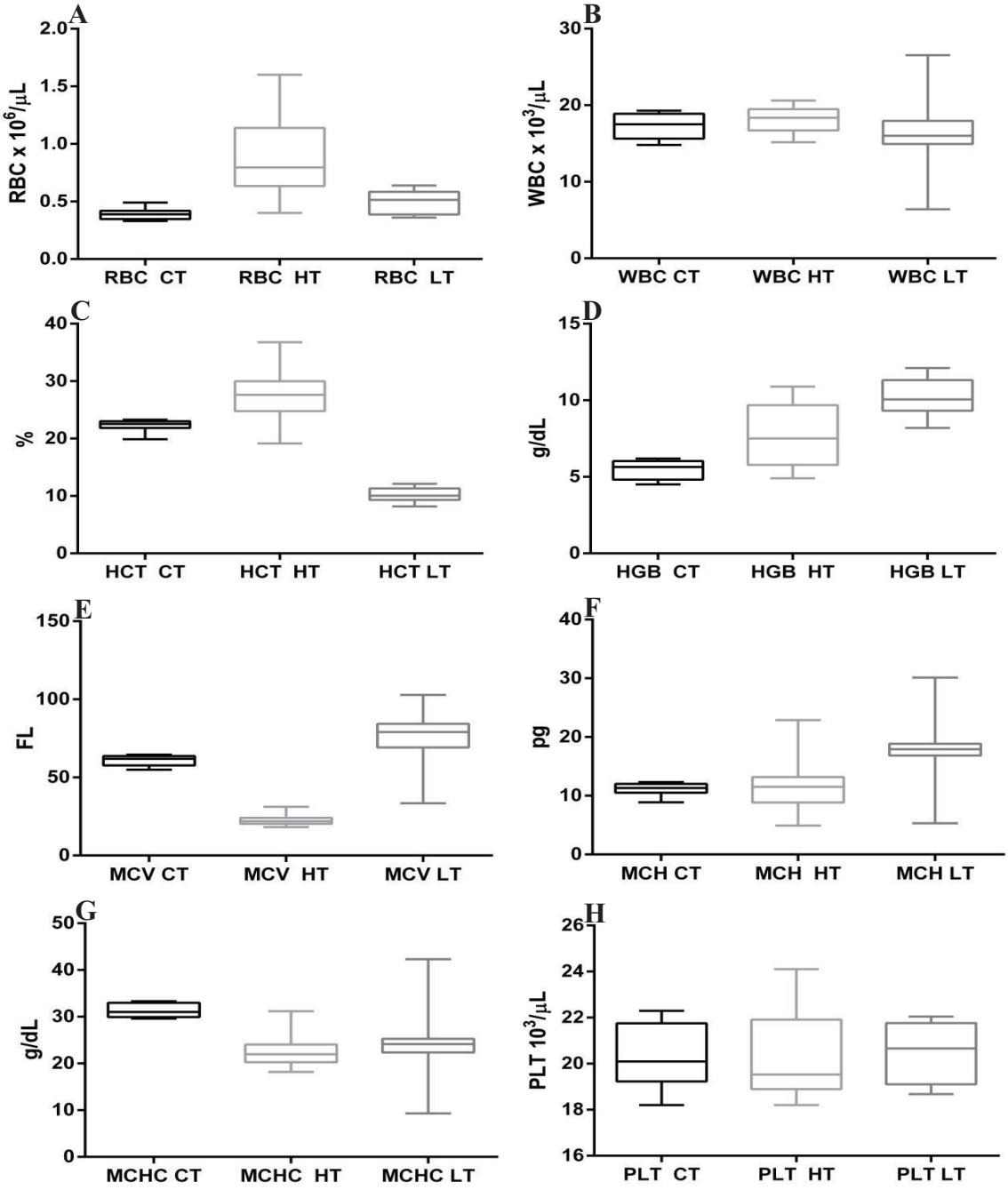

Figure 1. Mean values and standard deviation of number of erythrocytes (A), number of leukocytes (B), hematocrit (C), hemoglobin content (D), mean corpuscular volume (E), mean corpuscular hemoglobin (F), mean concentration of corpuscular hemoglobin (G) and platelets (H) of Brown bullhead at the control (normal) temperature (CT), high temperature (HT) and lower temperature (LT)

The increase in hematocrit is gradually made and directly proportional to the temperature. The obtained values are as follows: 6°C - Ht = 18.38±0.443%; 18°C - Ht = 22.30±0.317%; 31°C - Ht = 27.55±1.461%. Previous studies regarding blood analyzes of fish and other vertebrates have shown that when environmental temperature increases, the

volume of erythrocytes also increases (Gillooly and Zenil-Ferguson, 2014), directly affecting the hematocrit value. Hematopoiesis is also accelerated in cases of hyperthermia as a result of decreasing the erythrocyte-carrying capacity (Root effect), resulting the increase of hematocrit. The mechanism consists in activating circulating stress hormones (catecholamine) in hyperthermia and implicitly in hypoxia (Lai et al., 2006), which accelerate hematopoietic processes.

Platelets (PLT) represent the only hematological parameter that does not show any variation regardless of temperature intensity (PLT = 20.55±0.402 x 10^3/µL - 6°C; PLT = 20.30±0.434 x 10^3/µL - 18°C; PLT = 20.30±0.636 x 10^3/ML - 31 °C).

The erythrocyte count variation, as can be seen in Figure 1, also led to the change of the erythrocyte indices. Thus, the gradual increase in the number of erythrocytes is correlated (according to the calculation formula) with a gradual and directly proportional decrease of mean corpuscular volume (MCV) values. It is possible to observe a higher value of this index at low temperatures (6°C MCV = 76.33±5.779 fl), respectively a lower value in case of high temperatures (31°C MCV = 38.66±2.109 fl). The average value of mean corpuscular volume recorded at 18 °C (MCV = 60.90±1.077 fl) can be found between the highest and lowest values.

Mean corpuscular hemoglobin showed the lowest value under control conditions (18°C MCH = 11.1±0.345 pg), value close to that resulting from hyperthermia (31°C MCH = 11.56±1.522 pg). Under hypothermia, the mean corpuscular hemoglobin was higher (6°C MCH = 17.86±1.868 pg).

Mean corpuscular hemoglobin concentration showed the following mean values: 6°C - MCHC = 24.30±2.503 g/dL; 18°C - MCHC = 31.30±0.454 g/dL; 31°C - MCHC = 22.57±1.176 g/dL.

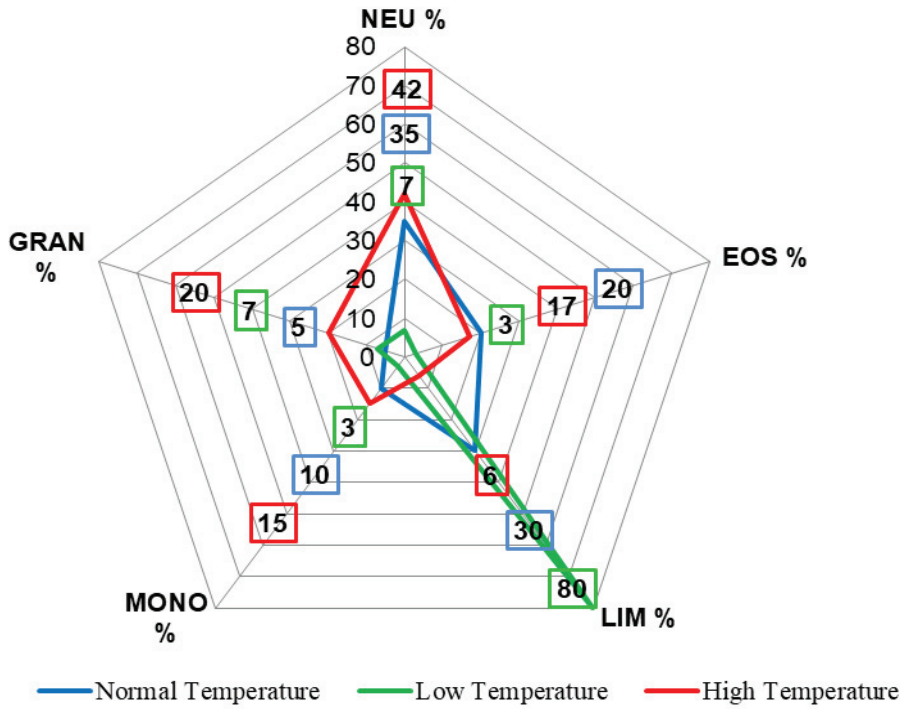

Figure 2. Leukocyte formula (%) in Brown bullhead (*Ameiurus nebulosus*) under different temperature conditions

Figure 2 graphically represents the leukocyte formula of brown bullhead subjected to thermal stress. Leukocytes are reported as percentage (relative values) and their values were obtained from smear readings. The graphic representation shows at 18°C the following values of the leucocyte formula: NEU = 35%; EOS = 20%; LYM = 30%; MONO = 10%; BAS = 5%. At 6°C, the leukocyte formula shows the following values: NEU = 7%; EOS = 3%; LYM = 80%;

MONO = 3%; BAS = 7%. The values for the leucocyte formula at 31°C are as follows: NEU = 42%; EOS = 17%; LYM = 6%; MONO = 15%; BAS = 20%.

The low percentage of neutrophils under hypothermia (6°C) can be correlated with the mean values obtained in the hematological profile (low RBC, low Ht) and an inefficient hematopoiesis at low temperatures (Kulkeaw et al., 2010). Moreover, from the leukocyte formula point of view, the extreme values were obtained under conditions of hypothermia, which means that the physiological status of the Brown bullhead is strongly influenced. In addition, in the case of hypothermia, lymphocytosis is observed, the percentage exceeding the maximum threshold of 40% (LYM = 80%, in this case).

The data presented in Table 1 (including the F values) reveal extremely significant differences ($p < 0.0001$) depending on the temperature for most determined blood biochemical parameters, except for total calcium (TC) where the difference among lots was insignificant ($p > 0.05$). Also, a significant difference ($p < 0.05$) was recorded in the case of glucose (BG).

Table 1. The mean values, multiple comparisons (ANOVA test) and the statistical significance for the metabolic profiles and the oxidative stress in situations of thermal stress in Brown bullhead (*Ameiurus nebulosus*)

Parameter	Abbr.	MU	LT*	NT*	HT*	F*	Sign.P
Proteic Profile							
Total Protein	TP	g/dL	0.98±0.04	3.30±0.33	2.36±0.38	15.58	<0.0001
Albumin	ALB	g/dL	2.34±0.05	1.30±0.13	1.53±0.21	13.55	<0.0001
Globulin	GLOB	g/dL	0.25±0.01	3.20±0.19	2.76±0.24	78.39	<0.0001
Blood Urea Nitrogen	BUN	mg/dL	10.33±0.52	4.00±0.30	7.74±0.41	56.51	<0.0001
Creatine	CREAT	mg/dL	0.79±0.05	0.27±0.01	0.71±0.07	27.36	<0.0001
Lipid Profile							
Cholesterol	CHOL	mg/dL	64.33±2.75	105.00±2.29	191.33±14.15	59.25	<0.0001
Triglyceride	TGC	mg/dL	100.33±2.51	329.00±9.69	282.33±25.47	58.47	<0.0001
Carbohydrate Profile							
Blood Glucose	BG	mg/dL	34.33±3.97	23.00±0.50	27.66±2.15	4.70	<0.05
Mineral Profile							
Total Calcium	TC	mg/dL	4.84±0.17	4.90±0.28	4.62±0.26	0.36	ns
Phosphorus	P	mg/dL	2.53±0.12	3.12±0.34	9.05±1.06	31.04	<0.0001
Blood Iron	Fe	µg/dL	283.18±0.23	97.27±10.74	230.23±30.53	17.46	<0.0001
Sodium	Na	mmol/L	129.00±1.75	111.70±2.02	121.33±1.38	25.04	<0.0001
Potassium	K	mmol/L	3.50±0.17	2.21±0.21	2.44±0.08	17.03	<0.0001
Enzyme Profile							
Alkaline Phosphatase	ALP	UL	65.33±3.02	155.00±3.13	121.66±6.93	91.9	<0.0001
Gamma-glutamyl transpeptidase	GGT	UL	16.80±1.1	2.00±0.21	18.66±0.48	149.1	<0.0001
Aspartate aminotransferase	AST	UL	1916.66±25.58	268.00±3.51	1448.00±147.8	96.13	<0.0001
Alanine aminotransferase	ALT	UL	14.66±0.57	14.00±0.36	39.10±4.17	34.33	<0.0001
Serum Amylase	AM EP	UL	135.66±9.04	44.00±2.49	84.00±2.70	66.5	<0.0001
Lactate Dehydrogenase	LDH	UL	1467.00±17.02	282.00±3.06	502.66±24.58	1319	<0.0001
Lipase	LIP	UL	295.66±4.73	55.00±3.14	124.00±12.95	230.4	<0.0001
Creatine Phosphokinase	CPK	UL	12024.33±265.4	17620.00±190.1	9523.00±393.7	197.1	<0.0001
Total Bilirubin	TB	UL	0.55±0.02	0.08±0.01	0.04±0.01	295.5	<0.0001
Oxidative Stress							
Superoxide Dismutase	SOD	U/gHGB	823.51±43.71	238.53±34.12	706.63±47.3	54.11	<0.0001

*LT – lower temperature (6°C); NT – normal temperature (18°C); HT – high temperature (31°C); F – F statistic

Protein profile parameters can vary depending on several factors: species, sex, age, water temperature, feed (Patriche et al., 2009). So, in our case, the variation of protein parameters was influenced only by water temperature, as the brown bullheads were of the same age and received the same type of feed. The same authors (Patriche et al., 2011) consider that the difference between the groups of circulating proteins in the blood does not necessarily represent a state of stress, this fact being attributed to the ratio of albumin to globulin, which should not fall below 0.3. Values obtained show the following albumin / globulin ratios: 6 °C ALB / GLOB = 9.35; 18°C ALB / GLOB = 0.406; 31°C ALB / GLOB = 0.554.

According to the same authors, a stress indicator valuable biomarker is the level of

glycaemia, where significant differences (P <0.05) were recorded between the three groups: 6°C BG = 34.33±3.97 mg/dL; 18°C BG = 23.00±0.50 mg/dL; 31°C BG = 27.66±2.15 mg/dL. Other experiments (Suljević et al., 2015) also demonstrate changes in blood glucose levels under thermic stress conditions. As for the mineral profile, the total calcium level does not differ significantly regardless of the water temperature (6°C TC = 4.84±0.17 mg/dL, 18°C TC = 4.90±0.28 mg/dL, 31°C TC = 4.62±0.26 mg/DL), as confirmed by other studies (Grigg, 1969; Cataldi et al., 1998). The mean values of the other mineral parameters show extremely significant differences between the three groups.

The values for iron (Fe) (6°C Fe = 283.18±0.23 µg/dL; 18°C Fe = 97.27±10.74 µg/dL; 31°C Fe = 230.23±30.53 µg/dL) may be correlated with blood hemoglobin level (6°C Hg = 10.2±0.388 g/dL, 18°C Hg = 5.5±0.196 g/dL, 31°C Hg = 7.66±0.638 g/dL), demonstrating that both low and high water temperatures lead to disturbances of the respiratory function because of peripheral vasoconstriction (low temperature) or lack of dissolved oxygen (high temperatures).

At an enzymatic level, significant differences were obtained between the three groups. The most dramatic differences were recorded in aspartate aminotransferase (6°C AST = 1916.66±25.58 U/L; 18°C AST = 268.00±3.51 U/L; 31°C AST = 1448.00±147.8 U/L), where in conditions of hypothermia, values approximately seven times higher appeared, and in hyperthermia conditions the standard value (at 18°C) was approximately five times higher. These values can conclude the occurrence of tissue lesions (Preston et al., 2016), AST being an enzyme found in several types of tissue (myocardium, liver, skeletal muscle, pancreas, kidney).

In situations of thermal stress, alkaline phosphatase (ALP) showed lower values compared to those obtained at 18°C (ALP = 155.00±3.13 U/L), suggesting a slight malnutrition of specimens exposed to low temperature (6°C ALP = 65.33±3.02 U/L), respectively to high temperature (31°C ALP = 121.66±6.93 U/L). This slight malnutrition is not the result of inappropriate feeding, because the three experimental groups have been provided with the same fodder conditions. Fish are poikilothermic organisms, so under conditions of temperature that do not fall within the limits of their biological comfort, they eat small amounts of food or can even refuse feed altogether. Ingestion and food intake are limited in high temperature conditions involving hypoxia (Saravanan et al., 2013). In fact, extreme temperatures have a negative influence on metabolic processes, which is why ALP has declined, but without reaching values that fall within pathological spectrum. Being involved in physiological calcium absorption processes (Villanueva et al., 1997), correlations can be made between total calcium (TC) and alkaline phosphatase (ALP) values in thermal stress situations.

Antagonist to ALP values, gamma-glutamyltransferase (GGT) presents elevated values in thermal stress situations, suggesting poor metabolic activity of the liver and a decrease in bile secretion. In the case of alanine aminotransferase (ALT), the values are elevated only under high temperature conditions (6°C ALT = 14.66±0.57 U/L, 18°C ALT = 14.00±0.36 U/L, 31°C ALT = 39.10±4.17 U/L). Serum amylase (AM EP) also showed elevated levels of thermal stress due to a pancreatic dysfunction or a possible renal insufficiency due to extreme temperatures. Danilenko et al. (1998), confirms changes in lactate dehydrogenase (LDH) values in fish depending on temperature, the same fact being confirmed by the results of our study (6°C LDH = 1467.00±17.02 U/L; 18°C LDH = 282.00±3.06 U/L; 31°C LDH = 502.66±24.58 U/L). Comparing the mean LDH values with the value obtained at 18°C in both hypothermia and hyperthermia, these are higher, resulting in extremely significant differences between the three groups (F = 1319; P <0.0001). LDH elevations may appear due to hypoxia (Panepucci et al., 2000) in the case of high water temperatures.

CONCLUSIONS

The results of our experiment indicate the fact that Brown bullhead has great plasticity and adaptability regarding environmental

conditions. Hematologic and metabolic profiles show changes when thermal stress is induced. No pathologic state was recorded during the experiment, but changes in behaviour were observed. Both for low temperature (6°C) and high temperature (31°C) fish reacted as a lethargic condition set off. We assume that Brown bullhead has a more complex mechanism in terms of thermal stress states.

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