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ALTERATIONS IN HEMATOLOGICAL, IMMUNOLOGICAL AND BIOCHEMICAL PARAMETERS OF TENCH, *TINCA TINCA* AFTER ACUTE ZINC EXPOSURE

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ABSTRACT

There has been an increasing ecological and global public health concern associated with zinc (Zn) pollution in aquatic habitats. Zinc is deposited mainly in aquatic sediments through adsorption and precipitation. Thus, benthic fish such as tench (*Tinca tinca*) are more vulnerable to Zn pollution. The present study was conducted to investigate the effects of acute high levels of Zn (20 mg/L for 24 hours) on the hematological, immunological and biochemical parameters of tench. It was found that clotting time (CT), thrombocyte count (TC), hemoglobin (Hb), sedimentation rate (SR), plasma total proteins, albumin and ceruloplasmin levels did not show any significant alterations. However, there was a significant increase in red blood cell (RBC) counts, hematocrit (Hct), plasma glucose and plasma lactate levels; and a sharp decrease in white blood cell (WBC) count and leucocrit (Lct) levels. The results of this study clearly indicate that, tench can be used as a responsive fish for monitoring the effects of zinc.

Keywords: Zinc, tench, heavy metal, blood, fish, indicator, hematology.

INTRODUCTION

The use of heavy metals such as zinc, in several industrial and technological applications such as electroplating and mining operations, increased exponentially. This, led to dramatic changes in the amount of zinc compounds released into the aquatic environments (Golding, 2006; Ergönül and Altındağ, 2011; Noulas *et al*., 2018). Zinc is a rare metal in nature, but it is commercially one of the most important metals in the world. Zinc concentration in runoff has been reported 1 to 15 mg/L (Golding, 2006) and the mean riverine fluxes of zinc to marine environment is reported to range between 20 to 200 kt/yr (Noulas *et al*., 2018).

Zinc is an essential element and has crucial functions in various physiological and biochemical processes (Tubek *et al*., 2008), however, it has toxic effects for fish at higher concentrations (Qu *et al*., 2014; Ciji and Nandan, 2014; McRae *et al*., 2016). Higher concentrations of Zn can alter ionoregulation (Loro *et al*., 2014), histopathological processes (Ciji and Nandan, 2014), and antioxidants (Qu *et al*., 2014), inhibit immune response (Witeska and Kosciuk, 2003; Kori-Siakpere and Ubogu, 2008; Ololade and Ogini, 2009), and interfere iron absorption (Hulten *et al*., 1991) in fish.

Fish have been an important source of protein, and they play a key role in the aquatic food webs (Pauly *et al*., 2000). Fish are very responsive to a broad range of contaminants, and any alterations in water quality can lead to important changes in the hematological and biochemical profile of fish (Remyla *et al*., 2008; Qu *et al*., 2014), Thus, they can be used as bioindicators of heavy metal pollution and environmental degradation (Shah and Altındağ, 2004; 2005; Ciji and Nandan, 2014; Authman *et al*., 2015). These parameters are easy to measure and they present integrated data on the physiological and biochemical alterations in fish (Remyla *et al*., 2008). Among the hematological parameters, hemoglobin (Hb) content, hematocrit (Hct) and red blood cell (RBC) counts are used to monitor impairment in gas exchange and stress (Shah and Altındağ, 2004; 2005); sedimentation rate (SR) may indicate disorders or infections (Svobodova *et al*., 1991; John, 2007; Jagtap *et al*., 2011). Similarly, leucocrit (Lct) and white blood cell (WBC) counts are used as a prognostic tool and early indicators of the alterations in the defense mechanism of fish (Brucka-Jastrzebska and Protasowiecki, 2005; Remyla *et al*., 2008). Thrombocytes (TC) have key functions in the coagulation of blood and in defense system of the organism (Passantino *et al*., 2005). Plasma glucose and lactate levels are common indicators used to evaluate the stress in fish (Yavuzcan and Ergönül, 2010; Ergönül *et al*., 2012). Plasma proteins have important roles in biological processes and they are very sensitive to environmental stressors. Therefore, they can be used as indicators of the health of fish (Lavanya *et al*., 2011). Ceruloplasmin is an important acute phase protein which activates under stressful conditions. It has many functions in the defense system such as restraining the dispersion of infectious agents (Sahoo *et al*., 2013; Sevcikova *et al*., 2011).

Tench (*Tinca tinca* L., 1758) is a commercially important species in some of the European countries and China (Wang *et al*., 2006). Furthermore, it is considered as a reliable test organism (Shah and Altındağ, 2004;

2005) for the contamination of sediments with heavy metals due to its bottom feeding behavior (Grosch *et al*., 2000). Since Zn is deposited mainly in sediments through adsorption and precipitation (Kadlec and Wallace, 2008), benthic fish such as tench are more vulnerable to Zn pollution. Thus, the present study was conducted to investigate the effects of an essential metal –Zn, on the hematological, immunological and biochemical parameters of tench and to understand the trends in these parameters in response to acute zinc treatment.

MATERIALS AND METHODS

Fish maintenance: Test fish, tench (*Tinca tinca* L., 1758) were obtained from Mogan Lake near Ankara, Turkey and brought in 50 L plastic cans to the laboratory. The experimental protocols used in this study were approved by the Ethical Committee of Ankara University. Fish were acclimated to laboratory conditions for 2 weeks in 800 L tanks. A 12-hour photoperiod was applied during the maintenance of the fish. Mean weight and length of the fish were 209.5±12.3 g and 25.2±3.1 cm, respectively. The tanks were aerated with air pumps and de-chlorinated tap water was used during experiments. The temperature, pH, electrical conductivity and dissolved oxygen levels were measured with a portable multi meter (WTW - Multi 3320). Total hardness and total alkalinity of the tap water were analyzed using standardized methods (APHA, 2012). Fish were fed twice a day with a commercial feed (45% crude protein; BioAqua.) at a rate of 2% body weight during the acclimation. Feeding was ceased 24 h prior to the blood sampling.

Bioassays: $ZnCl_2$ (Merck) was used to prepare stock solutions of zinc by diluting to the desired concentrations with distilled water. Test concentrations were adopted from Ergönül and Altındağ (2011) and fish were exposed to 20 mg/L $Zn^{2+}$for 24 h. Bioassays were performed under semi-static conditions and half of the aquaria water was renewed at 12 h intervals with the appropriate $ZnCl_2$ solution to keep the required concentration constant. Exposure tests were composed of two replicate tanks each containing 12 fish in 400 L tanks. Randomly selected 7 of the fish from each replicate tanks were removed after 24 h, and they were bled from the caudal vein using heparinized needles. These fish were used for determination of the blood cell counts and for obtaining plasma. The remaining 5 of the fish were used to determine clotting time by using non-heparinized plastic syringes. Control fish were tested in parallel and handled in the same way.

Hematological, Immunological and Biochemical analysis: The clotting time of the blood was determined according to Kawatsu and Sato (1987). The red blood cell (RBC), white blood cell (WBC) and thrombocyte (TC) counts were performed within 2 hours of blood collection by Improved Neubauer hemocytometer using Natt-Herrick as the diluent and stain (Svobodova *et al*., 1991). Hemoglobin (Hb) levels were determined with a commercial kit (Roche) according to the instructions supplied. Sedimentation rate (SR) of red blood cells, hematocrit (Hct) and leucocrit (Lct) measurements were made according to Svobodova *et al*. (1991) in micro capillary tubes. Blood was centrifuged at 14000 rpm for 5 min and plasma was subsequently aliquoted into eppendorf tubes and stored at -35°C until analysis. Plasma ceruloplasmin levels were measured with p-phenylene diamine (PPD) oxidase activity as described by Pelgrom *et al*. (1995). Plasma glucose, total protein and plasma albumin levels (Teco Diagnostics) and plasma lactate levels (Randox) were determined by using biochemical kits according to the instructions supplied.

Statistical analysis: Data were presented as mean±standard error (SE) of duplicate measurements. Normality and homogeneity of variances were controlled with preliminary analyses. Results were compared with One-Way ANOVA using the statistical packet program (SPPS v23.0) at a significance level of $P<0.05$.

RESULTS AND DISCUSSION

The physicochemical parameters (mean ±standard error) of the tap water used during bioassays are given in Table 1. Since no external aquarium heaters were used during the experiments, there was a slight fluctuation in water temperature depending on the ambient conditions.

Table 1. The physicochemical parameters (temperature, pH, electrical conductivity, dissolved oxygen, total hardness and total alkalinity of the tap water used during the bioassays.

Parameter	Mean ± SE
Temperature (°C)	17.7±4.47
pH	7.71±0.49
EC (µS cm^{-1})	217.47±16.95
DO (mg l^{-1})	6.39±0.45
Total hardness (mg l^{-1} $CaCO_3$)	77.5 ± 0.81
Total alkalinity (mg l^{-1} $CaCO_3$)	80 ± 0.96

During the experiments, one of the test fish were died and it was excluded from the analyses.

In the present study, no significant differences were found for clotting time and thrombocyte counts of fish exposed to acute high levels of zinc, compared to control (Figure 1a,b). The clotting time (sec.) of fish were 60.25±7.84 and 74.6±4.68; thrombocyte counts (cells 10^3 mm^{-3}) were 10.1±0.83 and 11.4±0.91 for exposure and control groups, respectively. Thrombocytes are involved in homeostasis and organisms defense. They are

produced mainly by the spleen and kidney in teleost fish and they play a central role in the intrinsic coagulation system (Jagadeeswaran *et al*., 1997). Thus, there is an important correlation between the number of blood thrombocytes and the clotting time in fish (Tavares-Dias and Oliveira, 2009). However, there is limited data on the effects of heavy metals on fish thrombocyte counts and clotting time. Witeska and Kosciuk (2003) reported dose and duration dependent deviations in thrombocyte counts in carp (*Cyprinus carpio*) exposed to Zn. Singh *et al*. (2008) found that clotting time increased in *Channa punctatus* exposed to sublethal concentrations of Cu. Witeska *et al*. (2006) found no significant differences in thrombocyte counts in tench exposed to Cd; Ololade and Ogini (2009) reported that thrombocyte counts did not show an obvious pattern in *Clarias gariepinus* exposed to sub-lethal concentrations of Zn.

It has been widely reported that heavy metals may lead to deviations in RBC indices of fish on a dose and duration dependent manner (Witeska and Kosciuk, 2003; Shah and Altındağ, 2004; 2005; Shah, 2006; Witeska *et al*., 2006; Ergönül *et al*., 2012). In the present study RBC counts (cells 10^6 mm^{-3}) and Hct (%) levels of fish exposed to zinc increased significantly (Figure 2a,b), however, Hb (g dl^{-1}) levels did not show any significant alterations (Figure 2c). The RBC counts of fish were 1.58±0.07 and 1.28±0.077, Hct levels were 31.43±1.67 and 26.4±1.08, Hb levels were 4.75±0.16 and 5.81±0.18 for exposure and control groups, respectively. The increase in the Hct levels of fish with a concurrent increase in RBC counts, seems to be a result of cell count increment rather than the swelling of the RBCs. Similar findings have been reported for *Catla catla* exposed to Zn (Remyla *et al*., 2008) and tench exposed to Cd (Witeska *et al*., 2006) and Hg (Shah and Altındağ, 2004). The release of RBCs into the blood stream via the contraction of spleen, is a well-known phenomenon during acute stress in fish (Schreck *et al*., 2016). However, it is also stated that hypoxia caused by gill damage and/or altered gaseous exchange at gills (Witeska *et al*., 2006) could lead to an increase in RBC counts. The increase in RBC counts did not correspond to an elevation in Hb levels, which could be due to disrupted Hb synthesis in newly formed RBCs because of iron deficiency caused by excess Zn. It is known that excess zinc may lead to decrease in the Fe levels in body (Hulten *et al*., 1991). Qu *et al*. (2014) found that zinc exposure led to a decrease in Fe levels in the liver of *Carassius auratus*. However, it should also be noted that Zn was found to stimulate erythropoiesis in fish (Chen *et al*., 2017).

Sedimentation rate (SR) is a measure of the speed of red blood cells that settle down in heparinized blood and is used to detect inflammation, disorders or infections (Svobodova *et al*., 1991; John, 2007; Jagtap *et al*., 2011). In the present study, the SR levels of fish exposed to Zn did not show any significant differences (Figure 2d). The SR levels (mm h^{-1}) were 3.32±0.24 for zinc exposed group and 3.48±0.14 for control fish. Kori-Siakpere *et al*. (2008) have reported a slight increase in SR in *Heteroclarias* sp. exposed to sublethal concentrations of Cd. Singh *et al*. (2008) observed an increase in SR in *Channa punctatus* exposed to Cu. Similar results were also recorded in *Salmo trutta fario* exposed to Co for 4 weeks (Atamanalp *et al*., 2010).

White blood cell count and Lct levels in fish can be used to detect the disturbances in the immunocompetence of fish exposed to toxicants (Brucka-Jastrzebska and Protasowiecki, 2005; Remyla *et al*., 2008; Schreck *et al*., 2016). They show great variation in fish, depending on the species, type of stressor, dose and duration (Shah and Altındağ, 2004; 2005; Ergönül *et al*., 2012). In the present study WBC counts and Lct levels of fish decreased significantly in exposure group (Figure 3 a,b). The WBC counts (cells 10^3 mm^{-3}) were 16.1±1.94 and 30.6±2.70, Lct levels (%) were 0.29±0.033 and 0.56±0.051 for exposure and control groups, respectively. Svobodova *et al*. (1994) reported a significant decrease in total WBC counts in *Cyprinus carpio* exposed to Zn. Witeska *et al*. (2006) reported a decrease in WBC counts and Lct level in tench exposed to cadmium. Kori-Siakpere and Ubogu (2008) recorded a slight decrease in WBC counts of *Heteroclarias* exposed to 10 mg/L Zn for 15 days. Ololade and Ogini (2009) found a gradual, dose dependent decrease in WBC counts of *Clarias gariepinus* exposed to Zn. Remyla *et al*. (2008) reported that WBC counts of *Catla catla* exposed to sub-lethal Zn concentrations did not show a marked difference. Witeska and Kosciuk (2003), found an initial increase during exposure and a gradual decrease in recovery period in *Cyprinus carpio* exposed to Zn. In present study, a sharp decrease was observed in WBC counts, which might be the result of direct toxic effects of Zn on WBCs. It is known that Zn may have cytotoxic effects on fish lymphocytes (Witeska and Kosciuk, 2003). A corresponding decrease was also observed in Lct levels; which indicates that the decrease was related to cell numbers rather than cell volumes.

The plasma glucose levels of fish generally show a sharp increase under stressful conditions to fulfill the rapid energy demand of fish (Witeska and Kosciuk, 2003; Martinez-Porchas *et al*., 2009; Yavuzcan and Ergönül, 2010; Ergönül *et al*., 2012). However, the magnitude depends on type of stressor and/or exposure time. In present study, a sharp increase was recorded in glucose levels of fish exposed to zinc. Plasma glucose levels (mg dl^{-1}) of fish exposed to zinc was 168.67±11.65 and 69.36±6.27 for control fish. A similar pattern was observed for plasma lactate levels. Plasma lactate levels (mmol l^{-1}) were 3.87±0.31 and 2.74±0.26 for exposure and control groups, respectively. Lactate is a closely related parameter to glucose metabolism and has been used as an indicator of anaerobic metabolism (Martinez-

Porchas *et al*., 2009). Heavy metals may cause hypoxia, due to altered ionic balance and/or enzyme activity at gills in fish (Witeska *et al*., 2006; Ergönül *et al*., 2012). Extended hypoxia may cause elevated levels of lactate, which is the final product of anaerobic glycolysis pathway (Hattink *et al*., 2006). Thus, it is reasonable to speculate that zinc exposure led to a shift from aerobic to anaerobic metabolism in tench after acute treatment with Zn.

Altered plasma total protein and albumin levels in fish may reflect the functional impairments in liver (Remyla *et al*., 2008). Albumin, is an important protein for the regulation of osmotic balance between plasma and tissues, synthesized in liver (Andreeva, 2010; Silva *et al*., 2015). The plasma total protein, albumin and ceruloplasmin levels did not show a marked difference (Figure 5a,b,c) in the present study for fish exposed to zinc. Plasma total protein levels (g l^{-1}) were 34.7±1.77 and 34.9±1.68, plasma albumin levels (g l^{-1}) were 12.1±0.82 and 12.8±0.83 and plasma ceruloplasmin levels (mg dl^{-1}) were 8.56±0.74 and 9.63±0.55 for exposure and control groups, respectively. Fırat and Kargın (2010) reported an increase in plasma total protein and albumin levels in *Oreochromis niloticus* exposed to Zn. Kori-Siakpere and Ubogu (2008) found a decrease in plasma total protein levels in *Heteroclarias* sp. exposed to Zn. Öner *et al*. (2008) observed that plasma total protein did not change in *Orechromis niloticus* exposed to Zn. The results of this study regarding the plasma total protein and albumin levels may indicate that acute high levels of Zn have no effect on the protein synthesis in tench. Ceruloplasmin is an acute phase protein and synthesized mainly in liver and increases during inflammation and tissue damage (Sahoo *et al*., 2013; Sevcikova *et al*., 2011). Ceruloplasmin levels of fish did not show any significant differences which may also indicate that Zn has no effect on the ceruloplasmin level in tench.

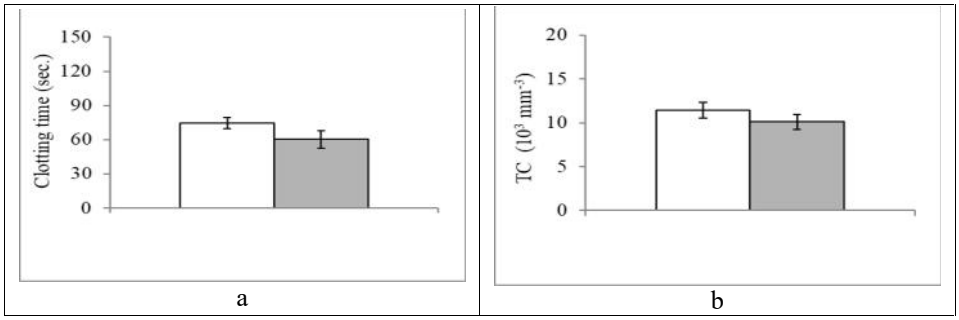

Figure 1. Clotting time (a) and thrombocyte counts (TC) (b) in tench exposed to 20 mg l^{-1} Zn^{2+} for 24 h. Light and dark bars represent control and exposure groups respectively, * P<0.05

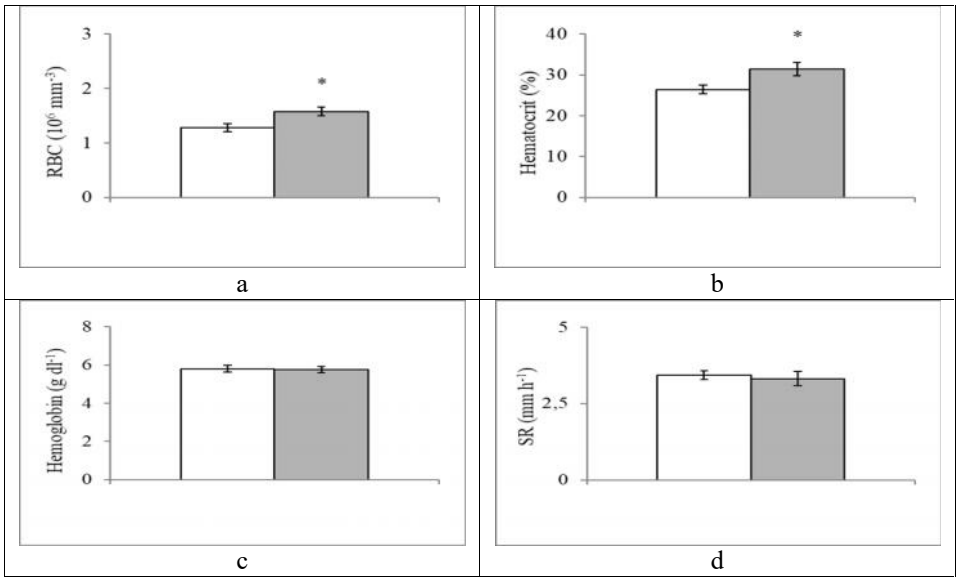

Figure 2. Red blood cell (RBC) counts (a), hematocrit (Hct) levels (b), hemoglobin (Hb) (c) and sedimentation rate (SR) (d) in tench exposed to 20 mg l^{-1} Zn^{2+} for 24 h. Light and dark bars represent control and exposure groups respectively, * P<0.05.

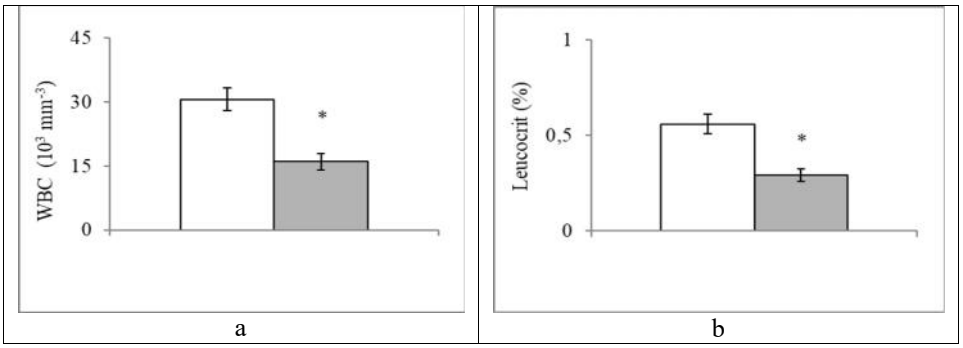

Figure 3. White blood cell (WBC) counts (a) and leucocrit (Lct) (b) levels in tench exposed to 20 mg l^{-1} Zn^{2+} for 24 h. Light and dark bars represent control and exposure groups respectively, * P<0.05

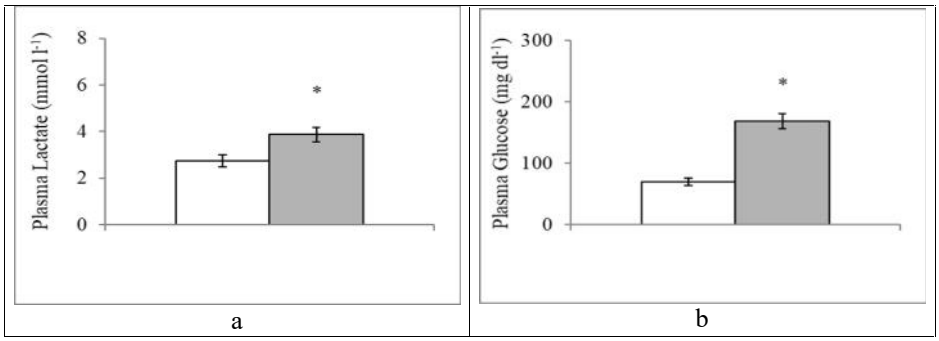

Figure 4. Plasma lactate (a) and plasma glucose (b) levels in tench exposed to 20 mg l^{-1} Zn^{2+} for 24 h. Light and dark bars represent control and exposure groups respectively, * P<0.05

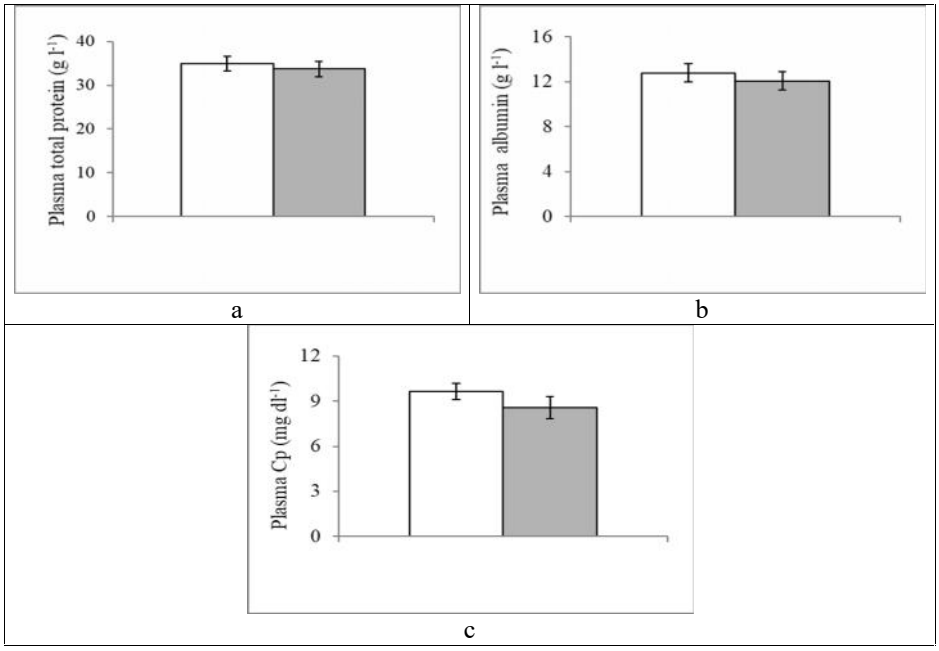

Figure 5. Plasma total protein (a), albumin (b) and plasma ceruloplasmin (c) levels in tench exposed to 20 mg l^{-1} Zn^{2+} for 24 h. Light and dark bars represent control and exposure groups respectively, * P<0.05.

Conclusion: Fish are highly sensitive to disturbances in environmental variables which may be reflected in hematological and biochemical parameters. Thus, the evaluation of blood parameters can be used as sensitive indicators for fish health (Shah and Altındağ, 2004; 2005; Remyla *et al*., 2008; Ergönül *et al*., 2012; Qu *et al*., 2014; Authman *et al*., 2015). Blood tests of fish allow us to establish the hematopoietic regulation of fish, and to

assess deviations from the normal range of blood parameters which can be used as potential indicators.

In conclusion, our results clearly indicate that the hematological and biochemical parameters of tench, a tolerant fish species, are sensitive to zinc intoxication. The blood parameters of tench including clotting time, TC, RBC and WBC counts, Hct, Lct, plasma glucose and proteins found sensitive to high levels of zinc. Thus, tench can be used as a responsive fish for monitoring the effects of trace elements such as zinc.

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