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BIOMARKERS OF OXIDATIVE STRESS AND ANTIOXIDANT DEFENSES IN THE BLOOD OF SEA TROUT (*SALMO TRUTTA* M. *TRUTTA* L.) AFFECTED BY ULCERATIVE DERMAL NECROSIS SYNDROM

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Skin ulcers on fish are one of the most well recognized indicators of polluted or otherwise stressed aquatic environments. In recent years, skin ulcer epidemics have been either experimentally or epidemiologically linked to exposure to a number of xenobiotic chemicals [29]. A lot of work was performed regarding skin ulcers in fish [10, 13, 25, 28, 29, 32, 33]. Aetiology of the outbreaks of skin ulcers remains unknown. There has been no conclusive evidence of the involvement of any particular organism as the primary pathogen [33]. It is suggested that the fungal infections are triggered by metabolites of the necrotic epidermal cells [14]. Skin samples were tested for standard virus isolation with negative results. Law (2001) suggests that skin ulcers in fish can have many different etiologies, including infectious agents, toxins, physical causes, immunologic causes, and nutritional and metabolic perturbations [25]. Ulcerative lesions are likely to be initiated by a series of factors that lead ultimately to a breach of the normal barrier function of the skin. Kane et al. (2000) reported that lesions in fish are associated with a variety of organisms including parasites and bacterial, viral, and fungal infectious agents. Trauma, suboptimal water quality, and other abiotic stress factors may result in the loss of homeostasis [13].

Patricia Noguera at “Workshop on salmonid skin diseases” gave a brief report about histological features of ulcerative dermal necrosis (UDN). Early lesions were described as thickening of epidermal cells with flattened upper surface, changes of nuclear morphology to karyolysis and pyknosis. Vesicle-like structures – due to the breakdown of the intercellular junctions – are leading to the separation of cells in the mid-layer [3]. The disease is characterized by an external, cotton-like appearance that radiates out in circular, crescent-shaped or whorled patterns with hemorrhagic necrotizing ulcers extending deep into the tissue. The initial signs of the disease are circles of pathologically-changed epidermis. Subsequently, the intercellular spaces dilate and communicate with

the exterior. Mycotic infection followed with subsequent invasion of the dermis and necrosis. Necrosis of the epidermal cells occurs simultaneously with fungal infections and marked responses of the melanophores [33]. The epidermal cells are shed and the fungus determines the further course of the disease which terminates in large ulcers covered with fungal hyphae [14]. UDN-like lesions affect of the head and dorsal areas, which within few days lead to a terminal fungal infection as the most obvious final observation before fish collapsed and died.

Řehulka (2002) obtained detailed information on the pathophysiological processes in the blood in cases of *Aeromonas*-induced UDN syndrome [32]. *Aeromonas* species as causative agents responsible for septicaemia, local inflammations and necroses on the skin, muscles and soft tissues [32]. The development of the skin lesions started as depigmented spots surrounded by a hyperaemic zone with the formation of ulcers, or the changes on the skin resembled furunculosis, taking the form of very large prominent bulges filled with clear exudate which, when broken, revealed haemorrhagically altered muscle. Some fish in his study showed exophthalmus; inflammation around pectoral fins; hyperaemia of the wall of the swim-bladder and petechial haemorrhages on the liver were found inside the abdominal cavity [32].

The potential of oxygen free radicals and other reactive oxygen species (ROS) to damage tissues and cellular components, called oxidative stress, in biological systems has become a topic of significant interest for environmental toxicology studies. The balance between prooxidant and antioxidant defenses (enzymatic and nonenzymatic) in biological systems can be used to assess toxic effects under stressful environmental conditions [40]. The depletion of antioxidant defense system and the changes in the activities of various antioxidant enzymes indicative of lipid peroxidation have been implicated in oxidative tissue damage. UDN syndrome seems to be quite capable of causing oxidative stress in liver, muscle, spawn and heart of sea trout [17–24, 38].

Objective of the present study was to examine the responses of oxidative stress biomarkers in the blood of male and female sea trout from control (healthy specimens) and UDN-affected trout from Słupia River (Northern Poland, Central Pomeranian region). Activities of antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT), glutathione reductase (GR), and glutathione peroxidase (GPx), ceruloplasmin (CP) level, and the total antioxidative capacity (TAC) were measured.

MATERIALS AND METHODS

Fish. Adult sea trout (*Salmo trutta* m. *trutta* L.), 3–5 years of age, were collected from site on the Słupia river, Słupsk, Northern Poland. Fish-catching took place in exact co-operation from Landscape Park “The valley of Słupia” as well as the Board of Polish Angling Relationship in Słupsk. Sea trout were

sampled from November to December, both in 2010 and 2011. Fig. 1 shows location of the river from which samples of sea trout were collected.

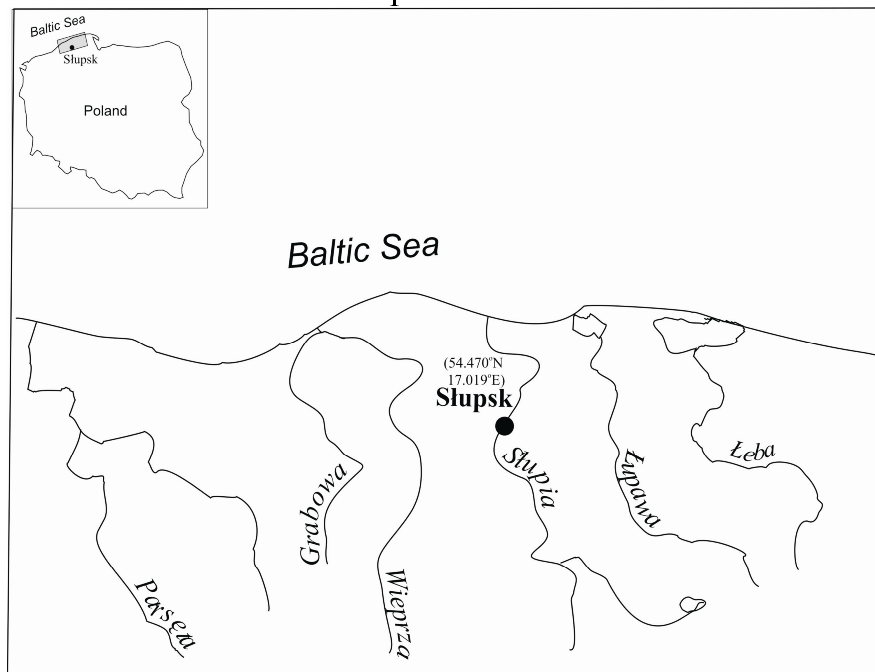

Fig. 1. Map of northern Poland. Marked is the Słupia river from which samples of sea trout were collected.

Sampling. The sampling for analysis from 27 healthy males and females (control group; Fig. 2A) as well as 26 males and females of sea trout affected by UDN syndrome (study group; Fig. 2B) was collected directly after catch. After catching, microbiological tests were carried out. These tests suggested that *Aeromonas hydrophila* complex caused UDN syndrome [37]. The pathogen was isolated from the infected sea trout.

A B

Fig. 2. Specimens of healthy trout (A) and UDN-affected trout (B).

Treatment of samples. Specimens in each group were dissected. One fish was used for each preparation. Blood samples (mean volume 5 mL) was taken from the caudal vein and immediately centrifuged at 3,000g for 10 min. The plasma was removed; the erythrocytes were washed three times with five volumes of physiological saline solution and centrifuged at 3,000g for 5 min. Erythrocytes' washing was repeated three times.

Chemicals. Thiobarbituric acid (TBA), oxidized and reduced glutathione (GSSG and GSH), NADPH, 5,5-dithiobis-2-nitrobensoic acid were purchased from Sigma. Ethylenediaminetetraacetic acid, thrichloroacetic acid, quercetin, hydrogen peroxide, ammonium molybdate, sodium aside, t-butylhydroperoxide, Tween-80, urea, 2,4-dinitrophenyl hydrazine (DNFH) were obtained from Fluka. All other chemicals were of analytical grade.

Analytical methods.

All enzymatic assays were carried out at $25 \pm 0.5°C$ using a spectrophotometer. The enzymatic reactions were started by the addition of the plasma or erythrocytes suspension, except for glutathione reductase (GR) and glutathione peroxidase (GPx) where hemolysate 1:20 was used. The specific assay conditions were as follows.

Superoxide dismutase (SOD, E.C. 1.15.1.1). SOD activity in the blood was measured spectrophotometrically by the Kostiuk et al. (1990) method using quercetin as a substrate after suitable dilution [16]. One activity unit was defined as the amount of enzyme necessary to produce a 50% inhibition of the quercetin (1.4 μM) reduction rates measured at 406 nm in 0 and 20th min. Activity is expressed in units of SOD per mL of blood.

Catalase (CAT, E.C. 1.11.1.6) assay. CAT activity was determined by measuring the decrease of H_2O_2 in the reaction mixture using a spectrophotometer at the wavelength of 410 nm by the method of Koroliuk et al. (1988) [15]. One unit of catalase activity is defined as the amount of enzyme required for decomposition of 1 μmol H_20_2/min per L of blood.

Glutathione reductase (GR, E.C. 1.6.4.2) assay. GR activity in the 1:20 hemolysate was assayed as described by Glatzle et al. (1974) by measuring the oxidation of NADPH at 340 nm [7]. The specific activity is expressed as μmol/min per mL of blood.

Glutathione peroxidase (GPx, E.C. 1.11.1.9) assay. The activity of GPx in hemolysate was measured spectrophotometrically following the method of Moin (1986) [27]. The rate of GSH oxidation was followed spectrophotometrically at 412 nm. GPx activity was expressed as μmol/min per mL of blood.

Ceruloplasmin (CP) level in the plasma was measured spectrophotometrically at the wavelength of 540 nm as described by Ravin (1961) [31]. CP content is expressed as mg per L of blood.

Total antioxidative capacity (TAC) assay. The TAC level in the blood was estimated spectrophotometrically at 532 nm following the method with ascorbate and ferric-induced Tween-80 oxidation to MDA formation [8]. TAC was expressed in %.

Lipid peroxidation level was determined by quantifying the concentration of TBARS, expressed as μmol of malondialdehyde (MDA) per L of blood, according to Kamyshnikov (2004) [12]. The μmol of MDA was calculated by

using $1.56 \cdot 10^5$ mM^{-1} cm^{-1} as molar extinction coefficient and lipid peroxides level in the blood was expressed in µmol of MDA per L of blood.

Oxidative modified proteins (OMP) assay. The rate of protein oxidation was estimated from the reaction of the resultant carbonyl derivatives of amino acids with 2,4-dinitrophenyl hydrazine (DNPH) as described by Levine et al. (1990) [26] in modification by Dubinina et al. (1995) [6]. The carbonyl content was calculated from the absorbance measurement at 370 nm and 430 nm and an absorption coefficient 22,000 $M^{-1} \cdot cm^{-1}$. Carbonyl groups were determined spectrophotometrically from absorbance at 370 nm (aldehyde derivates, OMP_{370}) and 430 nm (ketonic derivates, OMP_{430}), and expressed in nmol per mL of blood.

Statistical analysis. Results are expressed as mean ± S.E.M. All variables were tested for normal distribution using the Kolmogorov-Smirnov and Lilliefors test ($p > 0.05$). Significance of differences between the oxidative stress biomarkers contents and enzyme activities in the liver samples of sea trout (significance level, $p < 0.05$) was examined using Kruskal-Wallis one-way analysis of variance by ranks test. Correlations between parameters at the set significance level were evaluated using Spearman's correlation analysis [42]. All statistical calculation was performed on separate data from each individual with STATISTICA 8.0.

RESULTS

The values of lipid peroxidation for the males and females from control (healthy specimens) and UDN-affected trout are summarizing in Figure 1. TBARS levels of the plasma from trout infected by UDN syndrome was significantly higher than in the control group (healthy specimens), but no differences in erythrocytes lipid peroxidation levels between healthy and UDN-affected trout were found. UDN syndrome induced an increase of TBARS level in plasma of males (by 116%, $p = 0.003$) and females (by 164%, $p = 0.006$).

The effects of UDN infection on oxidatively modified proteins content, measured as carbonyl oxidation levels, in plasma are shown in Figure 2. UDN infection induced an increase in plasma carbonyl oxidation levels (aldehyde derivates) from males by 38% ($p = 0.000$) and by 47% ($p = 0.005$) from UDN-affected females. The carbonyl oxidation levels (ketonic derivates) in the plasma of males and females affected of UDN syndrome were significantly higher by 41% ($p = 0.000$) and by 49% ($p = 0.003$) than in values from control trout.

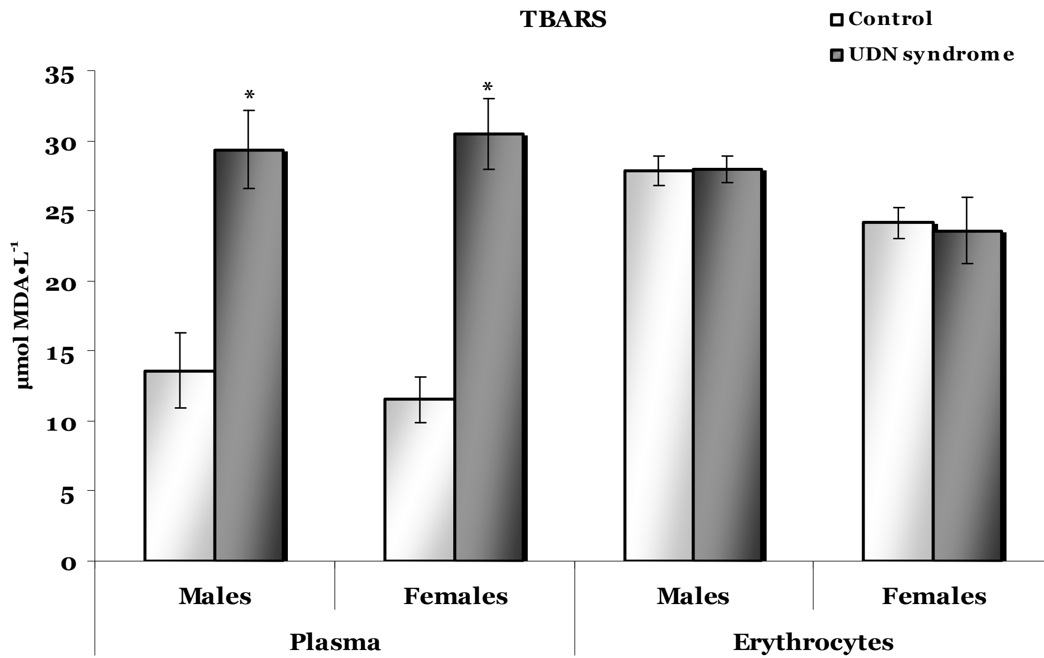

Fig. 1. TBARS levels (μmol MDA per L) in the plasma and erythrocytes from males and females of control (healthy specimens) and UDN-affected trout. Each value represents the mean ± S.E.M.

* The significant change was shown as $p < 0.05$ as compared to the control group values.

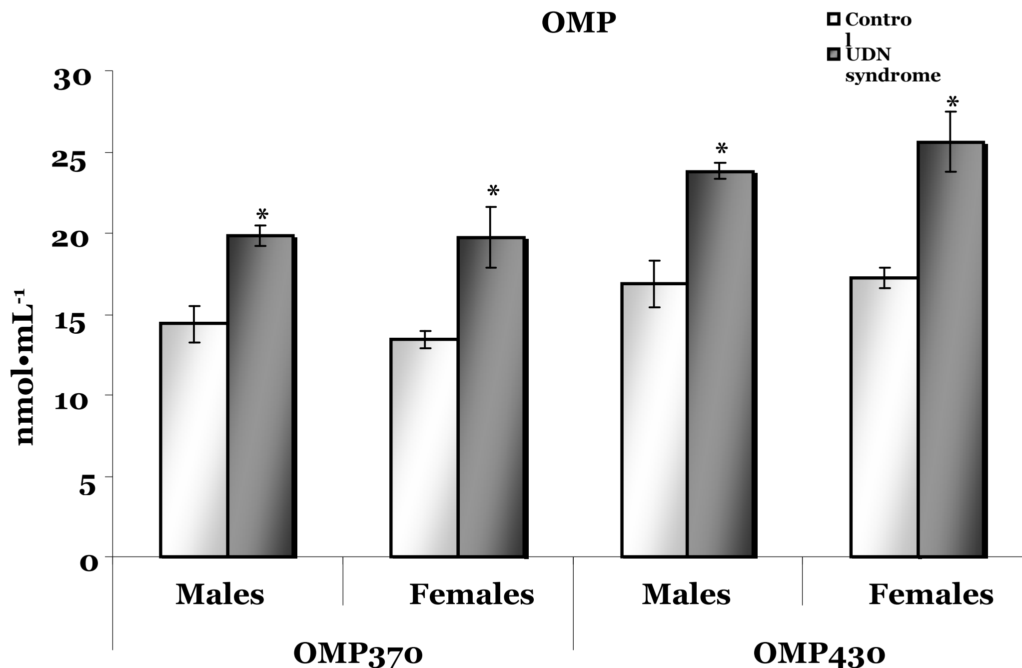

Fig. 2. Oxidatively modified proteins (OMP) content, measured by quantity of carbonyl oxidation (nmol per mL) in the plasma of males and females from control (healthy specimens) and UDN-affected trout.

Data are means ± S.E.M.

* The significant change was shown as $p < 0.05$ as compared to the control group values.

Activities of the antioxidant enzymes are shown in Table 1. No significant changes in blood SOD activity were found as a consequence of UDN infection only in males. SOD activity in the blood of UDN-affected females was increased by 49% ($p = 0.014$) compared to healthy females. CAT activity was decreased by 11.3% ($p = 0.000$) in blood of UDN-affected males and by 18.8% ($p = 0.006$) from females as compared to controls. UDN infection non-significantly affected GR activity (Table 1), which was inhibited by 11% ($p > 0.05$) in the blood of males and by 22% ($p < 0.001$) in females. Regarding the GPx (тable 1), its activity was significantly encreased by 84% ($p = 0.018$) in the blood of UDN-affected females as compared to the controls. UDN infection also increased CP level by 71% ($p = 0.015$) in females as compared to the controls. Regarding the total antioxidative capacity (Fig. 3), the UDN infection significantly decreased erythrocytes' TAC level by 23% ($p = 0.002$) both in UDN-affected males and females (by 13%, $p = 0.038$) as compared to the controls. Plasma TAC level was increased by 58.5% ($p = 0.002$) and by 83.4% ($p = 0.038$) in UDN-affected males and females, respectively.

Table 1. Activities of antioxidant enzymes in the blood from males and females of sea trout (*Salmo trutta* m. *trutta* L.) affected by ulcerative dermal necrosis (UDN) syndrome

Antioxidant enzymes activities	Males		Females	
	Control	UDN syndrome	Control	UDN syndrome
SOD, $U \cdot mL^{-1}$	587.52 ± 58.26	619.66 ± 46.53	431.57 ± 29.94	642.77 ± 63.85^b
CAT, $\mu mol \cdot min^{-1} \cdot L^{-1}$	12.40 ± 0.13	11.01 ± 0.25^a	12.32 ± 0.18	10.01 ± 0.36^b
GR, $nmol \cdot min^{-1} \cdot mL^{-1}$	172.31 ± 16.28	153.57 ± 9.89	178.60 ± 17.82	138.87 ± 25.50
GPx, $mmol \cdot min^{-1} \cdot mL^{-1}$	2.89 ± 0.37	2.96 ± 0.31	1.91 ± 0.22	3.52 ± 0.39^b
CP, $mg \cdot L^{-1}$	21.80 ± 2.26	29.07 ± 3.39	19.41 ± 2.75	33.25 ± 5.85^b

[a] difference is significant between control males and UDN-affected males ($p < 0.05$),
[b] difference is significant between control females and UDN-affected females ($p < 0.05$).

Several correlations between checked parameters were found. Plasma OMP_{430} level from UDN-affected males correlated positively with TBARS level both in plasma ($r = 0.444$, $p = 0.039$) and erythrocytes ($r = 0.597$, $p = 0.003$). Activities of some antioxidant enzymes (SOD and CAT) correlated with oxidatively modified proteins content in the plasma of males with UDN syndrome. CAT activity was connected inversely with GPx activity in the blood of UDN-affected males ($r = -0.649$, $p = 0.001$) and TAC ($r = -0.579$, $p = 0.005$). The relationships between blood SOD activity and both CAT activity ($r = -0.613$, $p = 0.012$) and TAC ($r = -0.537$, $p = 0.032$) were inverse (тable 2).

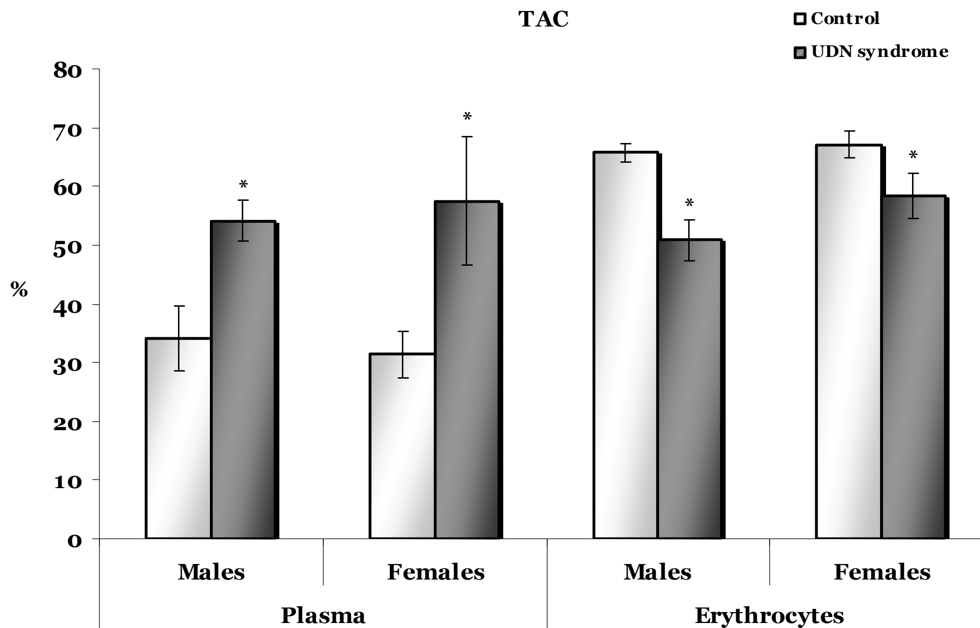

Fig. 3. The total antioxidative capacity (%) in the plasma and erythrocytes of males and females from control (healthy specimens) and UDN-affected trout. Data are means ± S.E.M.
* The significant change was shown as $p < 0.05$ as compared to the control values.

Table 2. Correlation analysis between oxidative stress biomarkers and antioxidative parameters in the blood from males and females of sea trout (*Salmo trutta* m. *trutta* L.) affected of ulcerative dermal necrosis (UDN) syndrome

Relation	Correlative coefficient, R	Significant difference level, p
	Males	
TBARS (plasma)–OMP_{430}, control	0.611	0.046
GR–OMP_{370}, control	-0.626	0.040
TBARS (plasma)–OMP_{430}, UDN syndrome	0.444	0.039
TBARS (er.)–OMP_{430}, UDN syndrome	0.597	0.003
TBARS (er.)–SOD, UDN syndrome	0.487	0.021
TBARS (er.)–TAC (er.), UDN syndrome	0.434	0.044
OMP_{370}–CAT, UDN syndrome	-0.492	0.020
OMP_{370}– TAC (er.), UDN syndrome	0.496	0.019
OMP_{430}–SOD, UDN syndrome	0.457	0.033
TAC (er.)–SOD, UDN syndrome	0.464	0.029
CAT–GPx, UDN syndrome	0.649	0.001
CAT–TAC (er.), UDN syndrome	-0.579	0.005
	Females	
SOD–CAT, control	-0.613	0.012
SOD–TAC (er.), control	-0.537	0.032
CAT–TAC (er.), control	0.862	0.000
GR–GPx, control	0.515	0.041
CP–TAC (plasma), control	0.589	0.016

DISCUSSION

This work focused on prooxidative changes and antioxidant enzymes activities in healthy and UDN-affected populations of sea trout. Lipid peroxidation and activities of antioxidant enzymes have been previously shown to vary considerably between fish species [1] and tissues [41]. Since all of the fishes in present study had been starved for equal time, the food deprivation effect is anticipated to be the same in all examined individuals. Sex is also a factor that might be important when measuring antioxidant enzymes and oxidative stress biomarkers. We did not find any differences between the sexes on antioxidant enzymes activities or in levels of protein modification, except in the erythrocytes' TBARS level ($p = 0.020$) and SOD activity ($p = 0.023$).

Certain conditions (such as disease, exposure to toxins, aging, exercise etc.) can increase the rate of oxidative damage, a condition called oxidative stress [30]. Oxidative stress has been defined as a disturbance in the balance between the production of reactive oxygen species (ROS), or free radicals and antioxidant defenses, which may lead to a series of biochemical and physiological changes, thus, altering normal body homeostasis and tissue injury [9]. The present study establishes that the blood of sea trout affected by UDN syndrome undergoes lipids and proteins oxidation due to the oxidizing effect of the ROS. Content of aldehydes, as end-products of lipid peroxidation assessed as TBARS, was increased in plasma both males and females (fig. 1). UDN syndrome induced the increase of TBARS levels only in the plasma. It appears that the UDN syndrome causes accumulation of end-product of lipid peroxidation in plasma of UDN-affected trout. Depletion of the cell antioxidant defense system was followed by the production of lipid and protein peroxidation products. The correlative relationships between oxidative stress biomarkers and activities of antioxidant enzymes also confirm this assumption (тable 2). Lipid hydroperoxide levels also appeared as potential markers of oxidative stress induced by UDN syndrome. TBARS level confirmed that the lipid peroxidation of starts rapidly in females and increase progressively to its maximum level in the plasma (fig. 1).

Bagnyukova et al. (2006) suggest the increase of protein carbonyls level, a set of products of free radical modification of proteins under stressful conditions [2]. OMP levels have risen as a result of other kinds of stresses [36]. Accumulation of oxidized proteins has also been found during aging and in some disorders [35]. Our results suggest, for the first time, that oxidative stress in the blood of sea trout may be mediated by UDN syndrome. Moreover, these results complete of the previous work in our laboratory reporting an inhibition of antioxidant defence system and activation of oxidative stress in the liver, heart and muscle of sea trout with UDN syndrome [17–24, 38].

It can be seen from the results, that UDN syndrome has caused modification of antioxidant enzyme activities in the blood of sea trout. They included SOD, CAT, GPx activity, as well as TAC level (тable 2, fig. 3). This

might be due to the modification of the above-mentioned enzymes by the end products of lipid and protein peroxidation. SOD, which occurs in animal cells both in the cytosol as a Cu/Zn enzyme and in the mitochondria as a Mn enzyme, may be of importance in preventing membrane lipid peroxidation when the latter is initiated by a combination of Fe^{3+} and $O_2^{-\cdot}$-generating system [4]. In our study, significant difference was observed in SOD activity of UDN-affected females. SOD is one of the several enzymatic systems often activated during oxidative stress and exposure to contaminants. The activation in SOD activity may result in cellular injury by superoxide radicals. This situation may reflect the positive correlation between SOD activity and TBARS level ($r = 0.487$, $p = 0.021$), as well as protein carbonyls level ($r = 0.457$, $p = 0.033$) in plasma of UDN-affected males (тable 2). CAT, associated with other enzymatic antioxidants (peroxidases, SOD) is capable of removing, neutralizing, or scavenging ROS and is, with the GSH redox cycle, the primary cellular enzymatic defense system against hydrogen peroxide (H_2O_2), that it converts to H_2O and O_2 [5]. The decreased CAT activities indicate the reduced capacity to scavenge hydrogen peroxide produced in the erythrocytes in response to UDN-induced oxidative stress.

Glutathione-mediated antioxidant defense system appears to be important in protecting cells against UDN-induced oxidative stress. The most important antioxidant enzymes in connection with lipid peroxidation are glutathione peroxidase, reductase, and transferase [11]. Inactivation of lipid-derived hydroperoxides can be catalyzed by GSH-dependent selenoperoxidases or certain non-seleno-GSH-S-transferases. Two selenoperoxidases are known to exist in cells: classical GSH-peroxidase (GPx), which acts on relatively polar substrates, e.g., H_2O_2 or fatty acid hydroperoxides, and phospholipid hydroperoxide GSH-peroxidase [39]. Glutathione peroxidase is dependent on access to glutathione disulfide by the NADPH-dependent enzyme glutathione reductase. Activation of glutathione-mediated antioxidant defense system results in oxidative stress and increased cytotoxicity, whereas elevation of intracellular GSH levels is recognized as an adaptive response to oxidative stress [34]. The GPx and SOD activities were significantly increased in the blood of UDN-affected females. These results suggest that both the glutathione-mediated antioxidant defense system and endogenous SOD play a critical role in intracellular antioxidant defence against UDN-induced oxidative stress. The importance of the glutathione-mediated antioxidant defense system in protection against oxidative stress was also demonstrated in rainbow trout adrenocortical cells [5]. CP level are important in maintaining the antioxidant defense properties of the blood. UDN-induced oxidative stress can be prevented by the action of non-enzymatic antioxidants such as CP. Correlation between CP content and TAC level in the plasma of healthy trout suggest this mention. Increase of CP level correlated with activation of the total antioxidative capacity under UDN-induced oxidative stress.

In conclusion, in both males and females, oxidative stress biomarkers in the blood from UDN-affected trout showed higher values as compared to the respective control. UDN induced an increase of TBARS level only in the plasma. Moreover, the increased of lipid and protein peroxidation biomarkers modifies antioxidant defense system and caused inhibition of CAT activity and erythrocytes' TAC level. Increased GPx activity and CP are important antioxidants against UDN-induced oxidative stress. This study encourages efforts to extend the knowledge of oxidative stress biomarkers for the identification of *Aeromonas* induced disorders and specific responses of fish typical of the UDN syndrome.

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BIOMARKERS OF OXIDATIVE STRESS AND ANTIOXIDANT DEFENSES IN THE BLOOD OF SEA TROUT (*SALMO TRUTTA* M. *TRUTTA* L.) AFFECTED BY ULCERATIVE DERMAL NECROSIS SYNDROM

Key words**:** *sea trout, Salmo trutta* m. *trutta* L.*, ulcerative dermal necrosis syndrome, oxidative stress, antioxidant defense system.*

Antioxidant defense system (activities of superoxide dismutase, catalase, glutathione reductase, and glutathione peroxidase, ceruloplasmin content, total antioxidative capacity), and oxidative stress biomarkers were determined in the blood of sea trout (*Salmo trutta* m. *trutta* L.) affected by ulcerative dermal necrosis (UDN) syndrome. In both males and females, lipid and protein oxidation in the blood from UDN-affected trout showed higher values as compared to the respective healthy specimens. The UDN syndrome induced an increase of thiobarbituric acid reactive substances (TBARS) levels only in the plasma and decrease in blood catalase activity. Both the glutathione-mediated antioxidant defense system and endogenous SOD play a critical role in intracellular antioxidant defense against UDN-induced oxidative stress. This study encourages efforts to extend the knowledge of oxidative stress biomarkers for the identification of *Aeromonas* induced disorders and specific responses of fish typical of the UDN syndrome.