www.trjfas.org
ISSN 1303-2712
Turkish Journal of Fisheries and Aquatic Sciences 14: 391-402 (2014) DOI: 10.4194/1303-2712-v14_2_09

Oxidative Stress Biomarkers in Liver of Sea Trout (*Salmo trutta* m. *trutta* L.) affected by Ulcerative Dermal Necrosis Syndrome

Halyna Tkachenko[1], Natalia Kurhaluk[1], Anastasiia Andriichuk[1], Elena Gasiuk[2], Sergiu Beschasniu[2]

[1] Pomeranian University in Slupsk, Institute of Biology and Environmental Protection, Department of Zoology and Animal Physiology, Department of Animals Physiology, Arciszewski Str. 22b, 76-200, Slupsk, Poland.
[2] Kherson State University, Department of Animal and Human Physiology, 40 Rokiv Zhovtnya Str. 27, 73-000, Kherson, Ukraine.

* Corresponding Author: Tel.: +4859.84 05360; Fax: +4859.84 05327;
E-mail: kurhaluk@apsl.edu.pl; tkachenko@apsl.edu.pl

Received 16 September 2013
Accepted 14 April 2014

Abstract

Antioxidant defense system and oxidative stress biomarkers were determined in the liver tissue from males and females of sea trout (*Salmo trutta* m. *trutta* L.) affected by *Aeromonas hydrofila* infection causing ulcerative dermal necrosis (UDN) syndrome. Histological results from hepatic parenchyma in UDN-affected trout show that cytoplasmic vacuolation of hepatocytes, loss of normal basophilia by cytoplasm, presence of pigment deposition in the parenchyma are a signs of liver deterioration. Impaired circulation in the liver of UDN-affected females manifests in oedema and an occurrence of a large number of erythrocytes in the sinusoids and the intercellular space. In both UDN-affected males and females, protein oxidation showed higher values as compared to the healthy trout. UDN syndrome caused a decrease of antioxidant enzyme activities. This might be due to inactivation of the above-mentioned enzymes by the end products of protein oxidation. The importance of the glutathione-mediated antioxidant defense system in UDN-induced oxidative stress was demonstrated. Results showed that UDN syndrome led to an oxidative stress in liver tissue able to increase of the oxidatively modified protein level.

Keywords: Sea trout, ulcerative dermal necrosis syndrome, oxidative stress, antioxidant defense system.

Introduction

During 2007-2012 years an obvious increase of moribund wild salmonids (brown and rainbow trout) with ulcerative dermal necrosis (UDN) syndrome was recorded in the Northern Pomerania region. A lot of work was performed regarding UDN syndrome in fish (Carbery and Strickland, 1968; Carbery, 1968, 1970; Roberts *et al.*, 1969; Munro, 1970; Klingler, 1974; Meier *et al*., 1977a, 1977b; Olivier *et al*., 1981; Johansson *et al*., 1982; McGarey *et al*., 1991; Law, 2001; Majumdar *et al*., 2007; Harikrishnan *et al*., 2009, 2010).

UDN syndrome is an epizootic fish disease characterized by the presence of severe, open dermal ulcers on the various regions of the fish (McGarey *et al*., 1991). There has been no conclusive evidence of the involvement of any particular organism as the primary pathogen (Roberts, 1993). Skin samples were tested for standard virus isolation with negative results (Roberts, 1993). The role of *Aeromonas hydrophila* and *A. sobria* in ulcerative disease syndrome is believed to be opportunistic or secondary and these bacteria are thought to play an important role in this degenerative disease (McGarey *et al*., 1991). The plasmid plays a pivotal role in the phenotype, growth and virulence of *A. hydrophila* and pathogenesis of aeromonad in ulcerative disease syndrome (Majumdar *et al*., 2007).

Affected fish may show exophthalmia, reddening of the skin and an accumulation of fluid in the scale pockets. The gills may haemorrhage and ulcers may develop on the dermis. Histopathologically, fish may exhibit epithelial hyperplasia in the foregut, leptomeningeal congestion in the brain, as well as a thrombosis and inflammation in the perisclerotic region and corneal epithelium of the eye (Fuentes and Perez, 1998). There may also be a severe branchitis, as indicated by leucocytic infiltration and dilation of the central venous sinus (Grizzle and Kiryu, 1993). Systemic infections are characterized by diffuse necrosis in several internal organs and the presence of melanin-containing macrophages in the blood (Ventura and Grizzle, 1988). Internally, the liver and kidneys are target organs. The liver may become pale, or have a greenish coloration, while the kidney may become swollen and friable. These organs are apparently

attacked by bacterial toxins and lose structural integrity (Huizinga *et al*., 1979). Even when tissue damage in the liver and kidneys is extensive, the heart and spleen are not necessarily damaged (Cipriano and Austin, 2011). Tissues such as skin and muscle have a limited morphological response to injury. The two most important phenomena that determine the outcome of cell injury appear to be critical cell membrane damage, with associated fluid and ionic imbalances, and inability of mitochondria, the powerhouse of the cell, to restart ATP synthesis (Law, 2001). The balance between oxidative stress biomarkers and antioxidant defenses in biological systems can be used to assess toxic effects of UDN syndrome.

The potential of 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 (Valavanidis *et al*., 2006). Oxidative stress results when production of ROS exceeds the capacity of cellular antioxidant defenses to remove these toxic species. Many environmental stressed factors engage signaling pathways that are activated in response to oxidative stress. The same sequences of events are also associated with the etiology and early pathology of many diseases (Limón-Pacheco and Gonsebatt, 2009). The depletion of antioxidant defense system and the changes in the activities of various antioxidant enzymes indicative of lipid and protein oxidation have been implicated in oxidative tissue damage (Limón-Pacheco and Gonsebatt, 2009). Our previously studies have shown that UDN syndrome seems to be quite capable to cause of oxidative stress in liver, muscle, heart, and spawn of brown trout (Kurhalyuk *et al*., 2009, 2010, 2011; Kurhalyuk *et al*., 2011; Tkachenko *et al*., 2011).

Objective of the present study is to examine the responses of oxidative stress biomarkers in the liver tissue of males and females of sea trout (*Salmo trutta* m. *trutta* L.) from healthy specimens and naturally 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), and the total antioxidative capacity (TAC), as well as oxidative stress biomarkers (thiobarbituric acid reactive substances (TBARS) and stable 2,4-dinitrophenyl hydrazine derivates of the oxidative modified carbonyl groups level) 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, during years 2007-2011. Figure 1 shows location of the river from which samples of sea trout were collected.

Sampling

The sampling for analysis from 73 and 87 healthy males and females (control group) as well as 122 males and 73 females of sea trout affected by UDN syndrome (study group) were collected directly after catch. After catching, microbiological tests were carried out. These tests suggested that *Aeromonas*

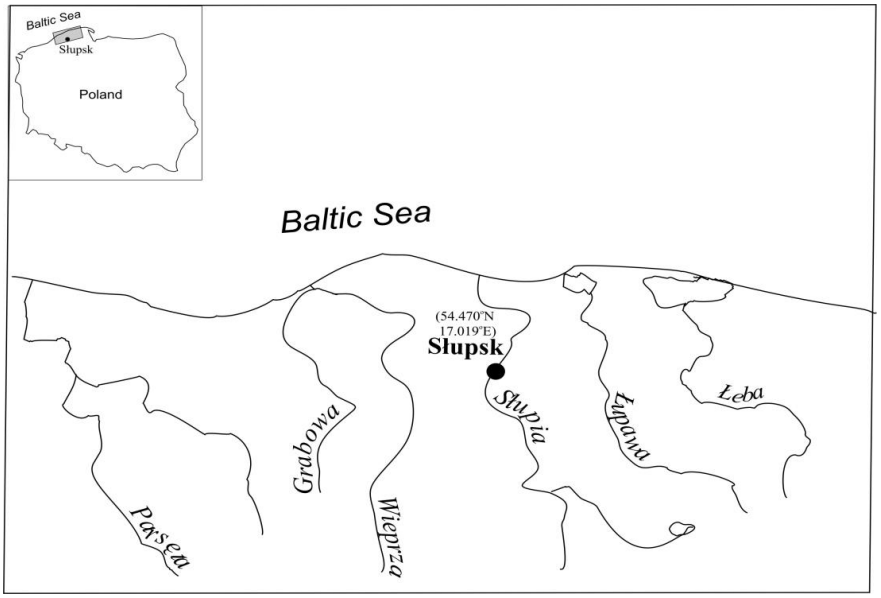

Figure 1. Map of northern Poland. Marked is the Słupia river from which samples of sea trout were collected.

hydrophila complex caused UDN syndrome (Szewczyk, 2005). The pathogen was isolated from the infected sea trout. Skin and gills samples were collected aseptically and washed three times with sterile saline. The organs were then put to buffer for obtaining the bacterial suspension (Dauner, 1967) and the 0.2 ml of suspension was inoculated into Aeromonas Isolation Agar with ampicillin at 37°C in triplicate. After 48 hours, green colony was re-isolated and subcultured on a new agar disc diffusion method (Bauer *et al*., 1966) on Mueller-Hinton agar supplemented with 1.5% NaCl.

Liver Homogenate Preparation

Specimens in each group were dissected. One fish was used for each preparation. Each liver sample was homogenized in cold Tris-HCl buffer (100 mM, pH 7.4) to obtain a 10% (w/v) liver homogenate. The homogenate was then centrifuged at 5,000×g for 15 min. Each supernatant was collected and stored at -20°C until analysis. The protein content of each sample was determined using Bradford method (1976) and bovine serum albumin as the standard.

Histological Assay

The liver tissues were fixed in a fresh solution of 4% paraformaldehyde (pH 7.4) at 4°C for 24 h and slides. Tissues were then placed in embedding cassettes dehydrated through graded ethanol, cleared in xylene and infiltrated with paraffin wax. Processed tissues were embedded in paraffin. Seven micron tissue sections were cut using a manual rotary microtome, stained with haematoxylin and eosin, mounted on glass slides and examined histologically. Images were obtained using a Micromed XS 5520 microscope (100/1.25x10) equipped with an eTREK DCM 320 3.0 M camera. Imaging and morphometry was examined using a graphical editor ImageJ (Image Processing and Data Analysis in Java).

Biochemical Assays

All enzymatic assays were carried out at 25±0.5°C using a Specol 11 spectrophotometer (Carl Zeiss Jena, Germany). The enzymatic reactions were started by adding the homogenate suspension. The specific assay conditions are presented subsequently. Each sample was analyzed in triplicate.

TBARS Assay for Lipid Peroxidation

Lipid peroxidation was determined as malondialdehyde (MDA) by estimation of the thiobarbituric acid reactive substances (TBARS) according to Kamyshnikov (2004). Reaction mixture containing 2.1 ml sub-sample of liver homogenate, 1.0 ml of 0.8% TBA, and 1.0 ml 20% TCA. The total volume was kept in a water bath at 100°C for 10 min. After cooling, mixture was centrifuged at 3,000 g for 10 min. The absorbance of the supernatant was measured at 540 nm. The TBARS level was expressed in nmol MDA per mg protein by using $1.56 \cdot 10^5$ mM^{-1} cm^{-1} as molar extinction coefficient.

Carbonyl Derivatives of Oxidatively Modified Protein (OMP) Assay

The rate of protein oxidative destruction was estimated from the reaction of the resultant carbonyl derivatives of amino acid reaction with DNFH as described by Levine *et al.* (1990) and as modified by Dubinina *et al.* (1995). DNPH was used for determining the carbonyl content in soluble and insoluble proteins. A quantity of 1 ml 0.1M DNPH (dissolved in 2M HCl) was added to 0.1 ml of the tissue samples after the protein was denatured. After adding the DNPH solution (or 2M HCl to the blanks), the tubes were incubated for 1 h at 37°C. The tubes were centrifuged for 20 min at 3,000 g. After centrifugation, the supernatant was decanted, and 1 ml of an ethanol-ethylacetate solution was added to each tube. Following the mechanical disruption of the pellet, the tubes were allowed to stand for 10 min and then centrifuged again (20 min at 3,000 g). The supernatant was decanted and the pellet was rinsed with ethanol-ethylacetate two times. After the final rinse, the protein was solubilized in 2.5 ml of 8 M urea solution. To speed up the solubilization process, the samples were incubated in a 95°C water bath for 10 min. The final solution was centrifuged to remove any insoluble material. 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 the difference in absorbance at 370 nm (aldehyde derivates, OMP_{370}) and 430 nm (ketonic derivates, OMP_{430}) and expressed in nmol per mg of tissue protein.

Superoxide Dismutase (SOD, E.C. 1.15.1.1) Activity Assay

SOD activity was measured with the method by Kostiuk *et al.* (1990). SOD activity was assessed by its ability to dismutate superoxide produced during quercetin auto-oxidation in an alkaline medium (pH 10.0). Briefly, 1.0 ml of C reagent was mixed with 0.1 ml liver homogenate (1:1,000). C reagent was made ex tempore (mixture of equal volumes of 0.1 M K,Na-phosphate buffer, pH=7.8 and 0.08M EDTA), pH of C reagent was adjusted to 10.0 by adding tetramethylenediamine. Distilled water (0.1 ml) was added to blank vials instead of liver homogenate. The total volume of all samples was brought up to 2.4 ml using distilled water. The reaction was initiated by adding 0.1 ml of quercetin (1.4 μM in dimethyl sulfoxide). Absorbance at 406 nm was measured immediately and after 20 min. Activity is expressed in

units of SOD per mg of tissue protein.

Catalase (CAT, E.C. 1.11.1.6) Activity 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). The reaction was initialized by adding 0.1 ml of liver homogenate into the incubation medium (2 ml of 0.03% solution of H_2O_2). The duration of this reaction was 10 min at room temperature. The reaction was terminated by rapid adding of 1.0 ml of 4% ammonium molybdate solution in 12.5 mM H_2SO_4 and 1 ml 125 mM H_2SO_4. Blank assay instead of liver homogenate included 0.1 ml of distilled water. All samples were centrifuged at 3,000 g for 5 min. The absorbance of the obtained solution was measured at 410 nm and was compared with that of the blank. One unit of catalase activity is defined as the amount of enzyme required for decomposition of 1 μmol H_2O_2 per min per mg of protein.

Glutathione Reductase (GR, E.C. 1.6.4.2) Activity Assay

GR activity in liver homogenate was measured according to the method described by Glatzle *et al.* (1974), with some modifications. The enzymatic activity was assayed spectrophotometrically by measuring $NADPH_2$ consumption. In the presence of GSSG and $NADPH_2$, GR reduces GSSG and oxidizes $NADPH_2$, resulting in a decrease of absorbance at 340 nm. The enzyme assay mixture contained 2.4 ml of 67 mM sodium phosphate buffer (pH 6.6), 0.2 ml of 7.5 mM oxidized glutathione, and 0.1 ml of liver homogenate. A control without $NADPH_2$ was used. Quantification was based on the molar extinction coefficient of 6.22 $mM^{-1} \cdot cm^{-1}$ of $NADPH_2$. GR activity was expressed as nmol NADPH per min per mg tissue protein.

Glutathione Peroxidase (GPx, E.C. 1.11.1.9) Activity Assay

GPx activity in liver homogenate was determined on the detection of nonenzymatic utilization of GSH as the reacting substrate at an absorbance of 412 nm after incubation with 5,5-dithiobis-2-nitrobenzoic acid (DTNB) according by the method of Moin (1986). Briefly, reaction mixture contained 0.8 ml of 0.1 M Tris-HCl buffer (pH 8.9) with 12 mM sodium azide, 6 mM EDTA, 0.2 ml of liver homogenate, 0.1 ml of 4.8 mM glutathione, and 0.1 ml of 20 mM t-butylhydroperoxide. The contents were incubated at 37°C for 10 min. The reaction was arrested by 0.2 ml of 20% TCA, and centrifuged. Supernatant was assayed for glutathione content by using Ellman's reagent (39.6 mg of DTNB in 10 ml of 1% sodium citrate). The rate of GSH reduction was followed spectrophotometrically at 412 nm. GPx activity is expressed as μmol GSH per min per mg tissue protein.

Total Antioxidant Capacity (TAC) Assay

The TAC level in the liver homogenate was estimated spectrophotometrically at 532 nm following the method with Tween 80 oxidation (Galaktionova *et al*., 1998). Briefly, 0.2 ml of liver homogenate was added to 2 ml 1% Tween 80. Blank assay instead of sample included 0.1 ml of distilled water. The mixture was incubated during 48 hours at 37°C. After cooling, 1 ml of TCA was added. The mixture was centrifuged at 3,000 g for 10 min. After centrifugation, 2 ml of supernatant and 2 ml of 0.25% TBA reagent was mixed. The mixture was heated in boiling water bath at 100°C for 15 minutes. The absorbance of the obtained solution was measured at 532 nm and was compared with the blank. TAC level was expressed in %.

Statistical Analysis

Results are expressed as mean±S.E.M. All variables were tested for normal distribution using the Kolmogorov-Smirnov and Lilliefors test ($P>0.05$). To assess the differences between the studied groups, Kruskal-Wallis one-way analysis of variance by ranks test was used (significance level, $P<0.05$). Correlations between parameters at the set significance level ($P<0.05$) were evaluated using Spearman's analysis (Zar, 1999). All statistical calculation was performed on separate data from each individual with STATISTICA 8.0 software (StatSoft, Poland).

Results

Histological structure of the liver tissue of healthy and UDN-affected males and females of sea trout is shown in Figure 2 and Figure 3. There were no statistically significant gender differences in the structure of the liver between healthy males and females (Figure 2A and Figure 3A). The liver had a tubular structure; the sections were presented in the form of balks. Veins and sinusoids between hepatic sections were present. Balks consisted of polygonal, adjacent cells. Hepatocytes were polygonal, often 5-6-sided shape, ranging in size from 26 to 30 microns. The cytoplasm of most hepatocytes was compact, fine-grained, with finely granular basophilic inclusions. The hepatocytes in most sections contain clear, pale staining nuclei with one to three nucleoli. Basophilic nucleus of sphericity or elliptical shape with a diameter of up to 10 microns was located in the center of the hepatocytes. The chromatin in the nucleus was well structured, distributed around nuclear membrane or near well-marked, large nucleoli.

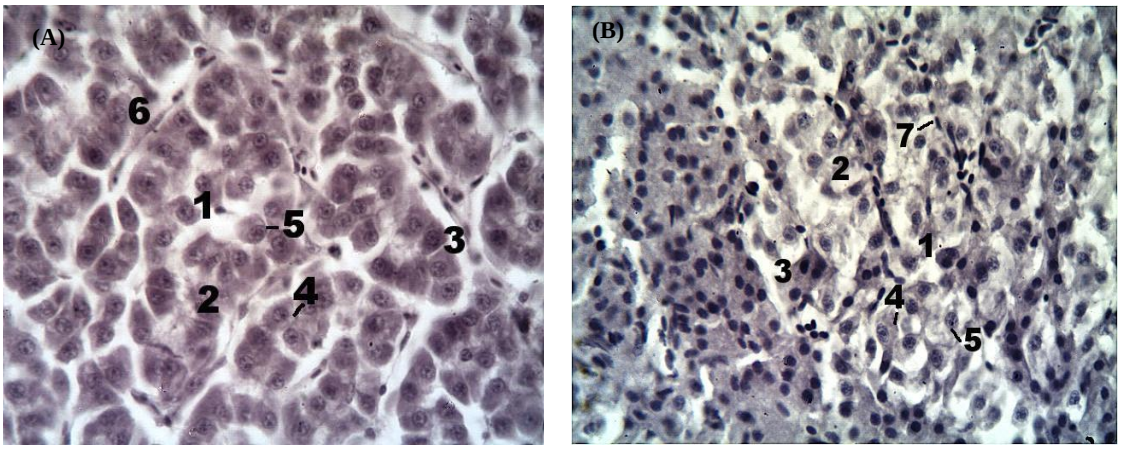

Figure 2. The structure of the liver parenchyma in the males of healthy (A) and UDN-affected (B) sea trout (1600x; Hematoxylin-eosin). 1 – vein, 2 – liver balks, 3 – sinusoids, 4 – hepatocyte, 5 – nucleus, 6 – endothelium, 7 – erythrocytes.

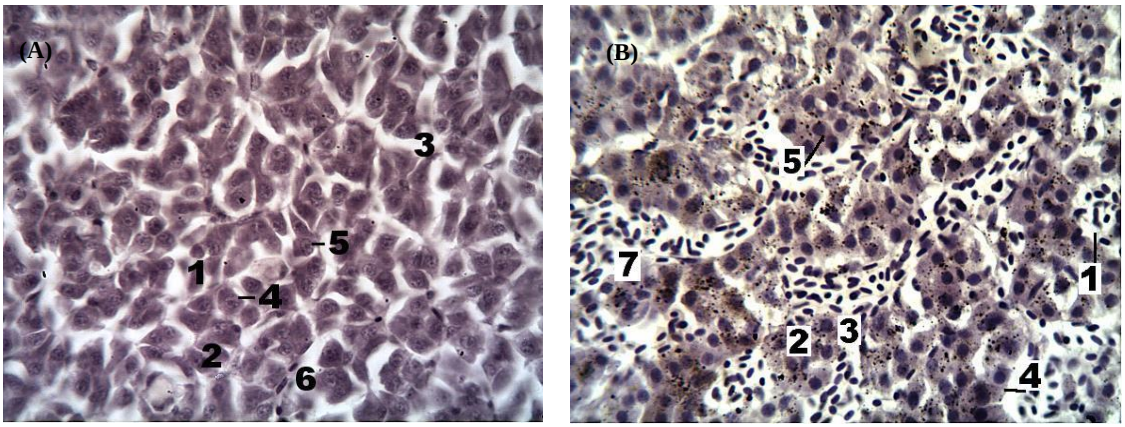

Figure 3. The structure of the liver parenchyma in the females of healthy (A) and UDN-affected (B) sea trout (1600x; Hematoxylin-eosin). 1 – vein, 2 – liver balks, 3 – sinusoids, 4 – hepatocyte, 5 – nucleus, 6 – endothelium, 7 – erythrocytes.

Morphological differences of liver preparations between UDN-affected trout and healthy individuals were observed (Figure 2B and Figure 3B). Liver parenchyma had a tubular structure with balks separated by veins and sinusoids. Balks were consisted of polygonal, adjacent hepatocytes. Intercellular spaces were greatly expanded due to edema. These changes are more pronounced in liver of females than in males (Figure 3B). Hepatic sinusoid and the intercellular spaces of the liver parenchyma were filled by erythrocytes. The cytoplasm of most hepatocytes was eosinophilic and foamy contained diffuse pockets of mild vacuolation. Basophilic nucleus sphericity or elliptical shapes with a diameter of 6 to 8 microns were located in the center of the hepatocyte. The well structured chromatin in the nucleus distributed around nuclear membrane or near large nucleoli. Number of nucleoli in the nucleus ranged from one to three. It should be noted decrease in the size of the nuclei in the liver of UDN-affected individuals. Some hepatocyte was no structural nuclei; they were represented by amorphous structureless mass. A small number of hepatocytes had two nucleuses after amitotic division or completes it (Figure 3B).

The oxidative stress biomarkers induced by the UDN syndrome was estimated through analysis of the lipid peroxidation and oxidatively modified proteins level (Figure 4 and Figure 5). The values of lipid peroxidation in the liver tissue in the males and females of control (healthy specimens) and UDN-affected trout are summarizing in Figure 4. No differences in TBARS level between healthy and UDN-affected trout were found.

The oxidative modification of protein could be used as molecular markers of oxidative stress (Levine *et al.* (1990). The oxidatively modified proteins content in the liver of UDN-affected trout, measured as carbonyl oxidation levels, are shown in Figure 5. UDN infection induced an increase of carbonyl oxidation levels (aldehyde derivates) of males by 45% (P=0.000) and by 12.8% (P=0.000) in the liver of UDN-affected females. The ketonic derivates of oxidatively modified protein levels both in males and females with UDN syndrome were significantly higher by 36.5% (P=0.000) and by 25.5% (P=0.000) respectively than in values from healthy trout.

Activities of the antioxidant enzymes are shown in Table 1 and Table 2. SOD activity was increased by 162% (P=0.000) of UDN-affected males as

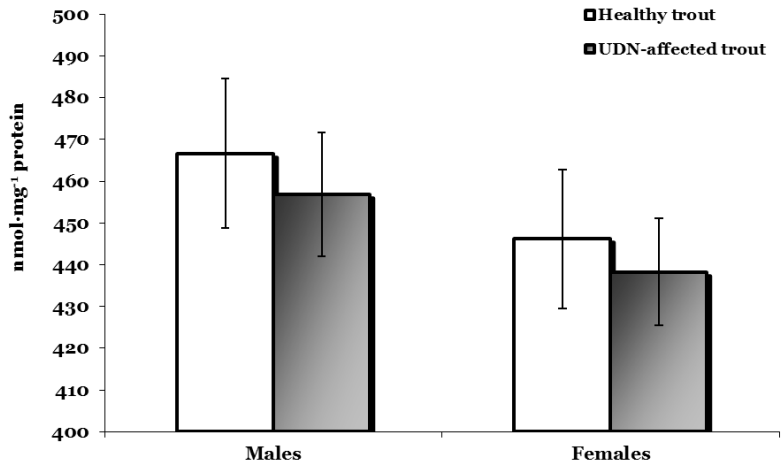

Figure 4. TBARS levels in the liver tissue of males and females of control (healthy specimens) and UDN-affected trout.
Each value represents the mean ± S.E.M. nmol·mg^{-1} protein.

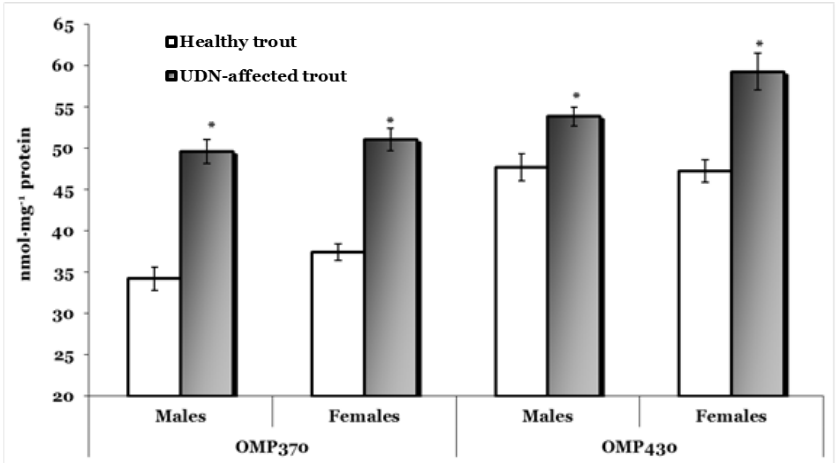

Figure 5. Oxidatively modified proteins (OMP) content, measured by quantity of carbonyl oxidation (aldehyde derivates, OMP_{370}; ketonic derivates, OMP_{430}) in the liver tissue of males and females from control (healthy specimens) and UDN-affected trout.
Data are mean ± S.E.M.
* The significant change was shown as P<0.05 as compared to the control group values.

Table 1. Enzymatic antioxidant defenses in the liver of UDN-affected males

Antioxidant enzymes activity	Healthy trout	UDN-affected trout
SOD, U·mg^{-1} protein	188.70±11.32	494.31±49.39*
CAT, µmol·min^{-1}· mg^{-1} protein	47.37±1.80	27.19±0.92*
GR, nmol GSH·min^{-1}· mg^{-1} protein	68.59±8.46	124.31±21.13
GPx, µmol $NADPH_2$·min^{-1}· mg^{-1} protein	119.06±9.37	112.30±14.86*

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

Table 2. Enzymatic antioxidant defenses in the liver of UDN-affected females

Antioxidant enzymes activity	Healthy trout	UDN-affected trout
SOD, U·mg^{-1} protein	194.15±10.56	217.96±18.82
CAT, µmol·min^{-1}· mg^{-1} protein	46.02±1.17	27.18±1.23*
GR, nmol GSH·min^{-1} mg^{-1} protein	139.32±8.49	71.98±10.61*
GPx, µmol $NADPH_2$·min^{-1} mg^{-1} protein	135.48±6.10	67.22±12.53*

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

compared to controls. No significant changes in liver SOD activity were found as a consequence of UDN-affected females. CAT activity was decreased by 42.6% (P=0.000) in the UDN-affected males and by 40.9% (P=0.000) in the females as compared to controls. UDN infection dramatically decreased GR activity, which was inhibited by 48.3% (P=0.000) in the males. Regarding the GPx, its activity was significantly decreased by 5.7% (P=0.000) in the UDN-affected males as compared to the controls, and by 50.4% (P=0.000) as compared to the UDN-affected females.

Regarding the total antioxidative capacity (Figure 6), the UDN infection significantly decreased TAC by 22.9% (P=0.000) in the liver tissue either UDN-affected males or females (by 33.8%, P=0.000) as compared to the controls.

Several correlations between checked parameters were found (Table 3). Activities of some enzymes (SOD, GR and GPx) correlated with aldehyde and ketonic derivates levels of oxidatively modified proteins in the males with UDN syndrome. Thus, CAT activity of UDN-affected females was connected with lipid peroxidation (r=0.316, P=0.006). The relationship between oxidatively modified proteins content and SOD activity was positive (r=0.250, P=0.033).

Discussion

This work focused on study of oxidative stress biomarkers and antioxidant enzymes activities in healthy and naturally UDN-affected populations of sea trout (*Salmo trutta* m. *trutta* L.) from Słupia river (Northern Pomerania region). Lipid peroxidation and activities of antioxidant enzymes have been previously shown to vary considerably between fish species (Aknes and Njaa, 1981) and tissues (Wdzieczak *et al*., 1982). 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. Therefore, we divided all individuals in our study to males and females. We found differences between the sexes on antioxidant enzymes activities (SOD, CAT, GPx, TAC), as well as aldehyde and ketonic derivates of oxidatively modified protein level (P=0.000).

Moreover, we also examined histopathological

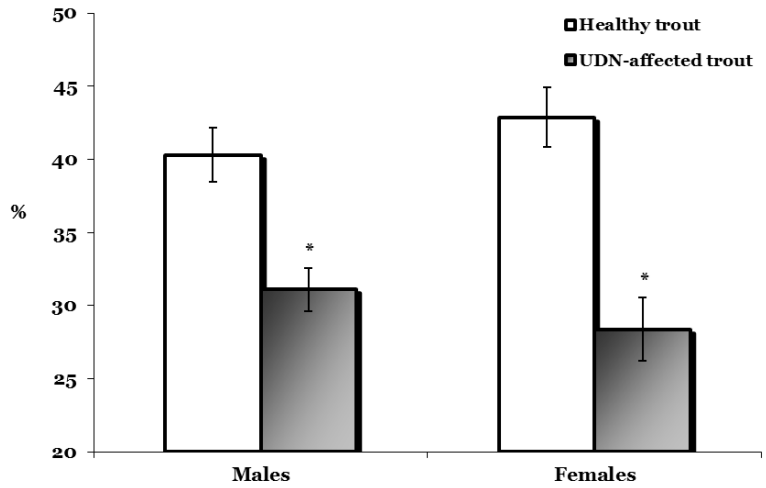

Figure 6. The total antioxidative capacity (%) in the liver of males and females from control (healthy specimens) and UDN-affected trout. Data are mean ± S.E.M. * The significant change was shown as P<0.05 as compared to the control values.

Table 3. Correlation coefficients between lipid peroxidation, oxydatively modified proteins levels and antioxidant defenses in the in the liver of UDN-affected males and females

Relation	Correlative coefficients, r	Significant difference level, P
	Males	
OMP_{370}–SOD	-0.272	0.020
OMP_{370}–GPx	0.283	0.015
OMP_{430}–SOD	0.344	0.003
OMP_{430}–GR	0.281	0.016
	Females	
TBARS–OMP_{370}	0.564	0.000
TBARS–CAT	0.316	0.006
OMP_{430}–SOD	0.250	0.033

changes in the liver tissue of UDN-positive males and females. The results of our studies provide evidence that ulcerative dermatitis associated with dystrophic injury of hepatic parenchyma. The progression of the disease affected the liver tissue in the following manner: loss of normal basophilia by cytoplasm, presence of vacuoles and pigment deposition in the parenchyma (Figure 3 and Figure 4). The differences in the size and shape of the liver cells, reducing the size of the nuclei, may be a symptom the general degeneration of the liver. However, the appearance of a small number of hepatocytes with amitotic nucleus in the liver of UDN-affected individuals may be the result of initial regenerative response. It is to increase relations between the surface of the nucleus and the cytoplasm at intensive impacts on the organism, such as the implementation of repair mechanisms in the injured liver. Furthermore, impaired circulation in the liver of UDN-affected trout (especially females) manifested in oedema and the occurrence a large number of erythrocytes in the sinusoids and the intercellular space.

Patricia Noguera at "Workshop on salmonid skin diseases" gave a brief report about histological features of UDN syndrome (Bruno *et al*., 2007). 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 (Bruno *et al*., 2007). 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 (Roberts, 1993). It is suggested that the fungal infections are triggered by metabolites of the necrotic epidermal cells (Khoo, 2000). The epidermal cells are shed and the fungus determines the further course of the disease which terminates in large ulcers covered with fungal hyphae (Khoo, 2000). 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.

Extensive studies have documented that *A. hydrophila* induce in UDN-infected fish focal haemorrhage, oedema, and dermal necrosis exposing the underlying muscle (Harikrishnan *et al*., 2009). For example, Olivier *et al.* (1981) were found that strains of *A. hydrophila* produced a dermonecrotic factor and were enterotoxigenic. Kozińska and Pękala (2012) suggest that *A. hydrophila* is the most versatile and dangerous species among fish pathogens from the genus *Aeromonas* and able to cause acute form of disease with septicaemia syndrome. Adhesion to various cells of fish organism may be the principal marker to detect virulent *Aeromonas* strains, which may cause specific disease spectrum (Kozińska and Pękala, 2012). Řehulka (2002) described the gross lesions of the skin and internal organs of the rainbow trout (*Oncorhynchus mykiss*) affected by *Aeromonas* strain. He suggest that *Aeromonas* infection caused depigmented erosions of various sizes mainly on the sides near the gill covers, the lesions were filled with clear to slightly turbid exudate, in some cases with a slight tint of blood and altered muscle. The muscle does not disintegrate under the skin bulge. The majority of the fish had pale gills, indicating different degrees of anaemia, exophthalmus and inflammations around the bases of the pectoral fins. Hyperaemia of the wall of the swim-bladder, splenomegalia, and petechial haemorrhages on the liver were found inside the abdominal cavity. The kidneys showed signs of albumin dystrophy and formation of hyaline droplets in the epithelium of proximal tubules. The wall of the macroscopically altered swim-bladder was thickened, congested and infiltrated edematous matter (Řehulka, 2002).

Harikrishnan *et al.* (2009) examine histo-pathological changes in lateral muscles, gills, liver, and heart in *A. hydrophila*-infected goldfish (*Carassius auratus*). The results of these studies provide evidence that *A. hydrophila* induce in *A. hydrophila*-infected fish appearance of a muscular haemorrhagic protuberance, which progressed into an extensive ulcerative dermatitis associated with focal haemorrhage, oedema, and dermal necrosis exposing the underlying muscle. The progression of the disease affected muscle, gills, liver, and finally the heart. Histopathological changes in *A. hydrophila*-infected liver were characterized by developed granulomatous inflammation and necrosis of hepatocytes (Harikrishnan *et al*., 2009). By day 18 after the infection were observed multiple fibromas and macrophage granulomas; by day 24 after the infection, liver of infected fish showed deep vacuolisation and granulation in the cytoplasm, pyknosis of the hepatocyte nuclei, necrosis, granulomatous inflammation, and large number of macrophages and fibroblasts. The liver exhibited focal necrosis of the hepatocytes with tubular degeneration of the intestinal microvilli and hepato-cellular necrosis by day 30 after the infection. By day 36 after the infection, liver tissues appeared oedematous and were congested with necrotic foci showing fibrin deposition or slight haemorrhage in the pulp, inflammation, free pre-granulomatous tissue, and mature granuloma (Harikrishnan *et al*., 2009).

Aydin and Ciltaş (2004) also described clinical abnormalities (abnormal movements, anorexia, darkening skin, pale and swollen gills, cherry-red coloured spleen, necrosis in liver, haemorrhage in kidney and serous exudates in intestine) of naturally

and experimentally infected by *A. hydrophila* juvenile rainbow trout (*Oncorhynchus mykiss*). Natural infection caused pathological changes in gill, brain, heart, kidney, liver and intestinal tissues (Aydin and Ciltaş, 2004).

Certain conditions (such as disease, exposure to toxins, aging, exercise etc.) can increase the rate of oxidative damage, a condition called oxidative stress (Pryor, 1986; Cross, 1987). 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 (Halliwell, 1994; Halliwell and Gutteridge, 1999). The present study establishes that the liver of sea trout affected by UDN syndrome undergo proteins oxidation due to the oxidizing effect of the ROS. Decrease in the cell antioxidant defense system followed by the production of oxidative modified protein products was noted. Aldehyde and ketonic derivates of protein degradation appeared as potential markers of oxidative stress induced by UDN syndrome.

It has been assumed that the oxidative stress as one of the important mechanisms of ROS effects (Nakazawa *et al*., 1996). Several studies have focused on the possible toxic effects of ROS on membrane components and identified a correlation between these effects and oxidative damage (Halliwell and Gutteridge, 1986; Yagi, 1993; Fridovich, 1998). These data suggest that altered composition of membranes may results in altered membrane integrity, permeability, and function. These would increase the susceptibility to lipid and protein oxidation. This cellular damage causes a shift in the net charge of the cell, changing the osmotic pressure, leading to swelling and eventually cell death (Nijveldt *et al*., 2001).

Bagnyukova *et al*. (2006) also suggest that stressful conditions may increase a level of oxidatively protein modification. OMP levels have risen as a result of other kinds of stresses (Stadtman and Levine, 2000). Accumulation of oxidized proteins has also been found during aging and in some disorders (Sohal, 2002). Our results suggest, for the first time, that oxidative stress in the liver of sea trout may be induced by UDN syndrome. Moreover, these results complete the previous study of our laboratory reporting an inhibition of antioxidant defense system and cumulation of oxidative stress biomarkers in the liver, heart and muscle of sea trout with UDN syndrome (Kurhalyuk *et al*., 2009, 2010, 2011; Kurhalyuk and Tkachenko, 2011; Tkachenko *et al*., 2011).

In the present study, the responses of UDN syndrome are probably not just dependent upon the increased oxidative stress but primarily on the modulation of antioxidant capacity (Table 1). It can be seen from our results that UDN syndrome caused a decrease in antioxidant enzyme activities in the liver of infected trout. They included CAT and GPx activities, as well as TAC level. This might be due to the inactivation of the above-mentioned enzymes by the end products of 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 (Cadenas *et al*., 1992). In contrast, no significant differences were observed for SOD activity in liver 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 and activation of enzyme by interaction with oxygen radicals. This situation may reflect the correlation between oxidatively modified protein and SOD activity in the liver of UDN-affected females ($r=0.250$, $P=0.033$). Correlation between TBARS level, derivatives of oxidatively modified proteins and SOD activity in the liver of UDN-affected males and females confirms our assumption (Table 2). Increased level of oxidatively modified protein level in females with UDN is correlated with enhanced SOD activity (Table 2). The enhancement SOD activity in our study suggest that probably its activation results from UDN linked mitochondrial $O_2^{\cdot-}$ production. As SOD provides the first line of antioxidant defense against produced mitochondrial superoxide, the increase in SOD activity shown in males and females, could reduce the exposure to superoxide and even to the hydroxyl radicals formed via the Haber-Weiss reaction. 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 (Dorval and Hontela, 2003). The decreased CAT activities in trout indicate the reduced capacity to scavenge hydrogen peroxide produced in response to UDN-caused 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 peroxidases, reductase, and transferase (Hayes and McLellan, 1999). Inactivation of lipid-derived hydroperoxides can be catalyzed by GSH-dependent seleno-peroxidases 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 (Ursini and Bindoli, 1987). Glutathione peroxidase is dependent on access to glutathione disulfide by the NADPH-dependent

enzyme glutathione reductase. Decrease 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 (Sagara *et al*., 1998). GR is not considered as a primary detoxificant of ROS, however GR recycles oxidized glutathione to its reduced form and has a central role in the glutathione dependent antioxidant protection. In addition, Tauler *et al.* (1999) suggested that ROS could preferentially activate GR because of the presence of more thiol groups in GR's structure. In our study, the activities of CAT and GPx were significantly decreased in the UDN-affected fish compared to the control group. These results suggest that both the glutathione-mediated antioxidant defense system and endogenous catalase play a critical role in intracellular antioxidant defenses against UDN-induced oxidative stress. Our assumption is consistent with the correlative analysis where UDN-induced oxidative stress resulted in modification of GR, GPx, and CAT activity (Table 2). The importance of the glutathione-mediated antioxidant defense system in protection against oxidative stress was also demonstrated in rainbow trout adrenocortical cells (Dorval and Hontela, 2003).

In conclusion, in both males and females, carbonyl derivates of oxidatively modified proteins in the liver of UDN-affected trout showed higher values as compared to the respective healthy controls. Moreover, increased of protein oxidation biomarkers modifies antioxidant defense system and caused inhibition of GPx and CAT activity, as well as TAC level. This study encourages efforts to extend the knowledge of oxidative stress biomarkers for the identification of *Aeromonas* spp. induced disorders and specific responses of fish typical of the UDN syndrome.

Acknowledgments

This study was funded by the Departmental Grant for Young Scientists of Pomeranian University in Slupsk. This study was also supported by The International Visegrad Fund (N51200912).

References

Aksnes, A. and Njaa, L.R. 1981. Catalase, glutathione peroxidase and superoxide dismutase in different fish species. Comp. Biochem. Physiol., 69(B): 893-896. doi: 10.1016/0305-0491(81)90402-8

Aydin, S. and Ciltaş, A. 2004. Systemic infections of *Aeromonas hydrophila* in rainbow trout (*Oncorhynchus mykiss* Walbaum): gross pathology, bacteriology, clinical pathology, histopathology and chemotherapy. J. Anim. Vet. Adv., 3: 810-819.

Bagnyukova, T.V., Chahrak, O.I. and Lushchak, V.I. 2006. Coordinated response of goldfish antioxidant defenses to environmental stress. Aquatic Toxicol., 78(4): 325-331. doi:10.1016/j.aquatox.2006.04.005

Bauer, A.W., Kirby, W.M., Sherris, J.C. and Turck, M. 1966. Antibiotic susceptibility testing by a standardized single disk method. Am. J. Clin. Pathol., 45: 493-496.

Bradford, M.M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem., 72: 248-254. doi: 10.1006/abio.1976.9999

Bruno, D., Crumlish, M., LaPatra, S., Noguera, P. and Verner-Jeffreys, D. 2007. Workshop on salmonid skin diseases. European Association of Fish Pathologists 13th International Conference on Fish and Shellfish Diseases, 18th September 2007, Grado, Italy: 7-10.

Cadenas, E.P., Hochstein, P. and Ernster, L. 1992. Pro- and antioxidant functions of quinones and quinone reductases in mammalian cells. Adv. Enzymol., 65: 97-146. doi: 10.1002/9780470123119.ch3

Carbery, J.T. 1968. Ulcerative dermal necrosis (UDN) of salmon: description, etiology and differential diagnosis. Bull. off Int. Epizoot., 69: 1401-1410.

Carbery, J.T. 1970. Observations on blood parameters of brown trout with ulcerative dermal necrosis. Res. Vet. Sci., 11: 491-493.

Carbery, J.T. and Strickland, K.L. 1968. Resistance of rainbow trout to ulcerative dermal necrosis. Nature, 217: 1158. doi: 10.1038/2171158a0

Cipriano, R. and Austin, B. 2011. Furunculosis and other Aeromonad Diseases. In: P.T.K. Woo and D.W. Bruno (Eds.), Fish Diseases and Disorders. Viral, Bacterial and Fungal Infections, Bacteriology Group Leader, Marine Scotland, Aberdeen, UK: 424-484.

Cross, C.E. 1987. Oxygen radicals and human disease. Ann. Intern. Med., 107: 526-545. doi: 10.7326/0003-4819-107-4-526

Daubner, I. 1967. Mikrobiologia vody (Microbiology of water). Slov. Akad. Vied., Bratislava, 345 pp. (In Slovak).

Dorval, J. and Hontela, A. 2003. Role of glutathione redox cycle and catalase in defense against oxidative stress induced by endosulfan in adrenocortical cells of rainbow trout (*Oncorhynchus mykiss*). Toxicol. Appl. Pharmacol., 192: 191-200. doi: 10.1016/S0041-008X(03)00281-3

Dubinina, E.E., Burmistrov, S.O., Khodov, D.A. and Porotov I.G. 1995. Oxidative modification of human serum proteins. A method of determining it. Vopr. Med. Khim, 41: 24-26 (In Russian, English abstract).

Fridovich, I. 1998. Oxygen toxicity: a radical explanation. J. Exp. Biol., 201: 1203-1209.

Fuentes, R.J.M. and Perez, H.J.A. 1998. Isolation of *Aeromonas hydrophila* in the rainbow trout (*Oncorhynchus mykiss*). Veterinaria Mexico, 29: 117–119.

Galaktionova, L.P., Molchanov, A.V., Elchaninova, S.A. and Varshavskiy, B.Y. 1998. The lipid peroxidation processes in patients with ulcerous illness of stomach and duodenum. Clin. Lab. Diagnostics, 6: 10-14 (In Russian).

Glatzle, D., Vuilleumier, J.P., Weber, F. and Decker, K. 1974. Glutathione reductase test with whole blood, a convenient procedure for the assessment of the riboflavin status in human. Experientia, 30: 665-667. doi: 10.1007/BF01921531

Grizzle, J.M. and Kiryu, Y. 1993. Histopathology of gill, liver and pancreas and serum enzyme levels of channel catfish infected with *Aeromonas hydrophila* complex. Journal of Aquatic Animal Health 5: 36–50.

Halliwell, B. 1994. Free radicals, antioxidants and human disease: Curiosity, cause or consequence. Lancet, 344: 721-724. doi: 10.1016/S0140-6736(94)92211-X

Halliwell, B. and Gutteridge, J. 1986. Oxygen free radicals and iron in relation to biology and medicine: some problems and concepts. Arch. Biochem. Biophys., 246: 501-514. doi: 10.1016/0003-9861(86)90305-X

Halliwell, B. and Gutteridge, J.M. 1999. Free Radicals in Biology and Medicine. Third ed., Oxford University Press, New York. 3rd Ed., Oxford University Press, New York, 878 pp.

Harikrishnan, R., Balasundaram, C., Moon, Y.G., Kim, M.C., Kim, J.S., Dharaneedharan, S. and Heo, M.S. 2010. Phytotherapy of ulcerative dermatitis induced by *Aeromonas hydrophila* infection in goldfish (*Carassius auratus*). Acta Vet. Hung., 58: 29-37. doi: 10.1556/AVet.58.2010.1.3

Harikrishnan, R., Balasundaram, C., Moon, Y.G., Kim, M.C., Kim, J.S. and Heo, M.S. 2009. Use of herbal concoction in the therapy of goldfish (*Carassius auratus*) infected with *Aeromonas hydrophila*. Bull. Vet. Inst. Pulawy, 53: 27-36.

Hayes, J.D. and McLellan, L.J. 1999. Glutathione and glutathione-dependent enzymes represent a coordinately regulated defense against oxidative stress. Free Rad. Res, 31: 273-300.

Huizinga, H.W., Esch, G.W. and Hazen, T.C. 1979. Histopathology of red-sore disease *(Aeromonas hydrophila)* in naturally and experimentally infected largemouth bass *Micropterus salmoides* (Lacepede). Journal of Fish Diseases, 2, 263–277. doi: 10.1111/j.1365-2761.1979.tb00169.x

Johansson, J.K., Svensson, K. and Eridberg, G. 1982. Studies on the pathology of ulcerative dermal necrosis (UDN) in Swedish salmon, *Salmo salar* L., and sea trout, *Salmo trutta* L., populations. J. Fish Diseases, 5: 293-308. doi: 10.1111/j.1365-2761.1982.tb00485.x

Kamyshnikov, V.S. 2004. Reference book on clinic and biochemical researches and laboratory diagnostics. MEDpress-uniform, Moscow (In Russian).

Khoo, L. 2000. Fungal diseases in fish. Sem. Avian Exotic Pet Med, 9: 102-111. doi: 10.1053/AX.2000.4623

Klingler, K. 1974. Ulcerative dermal necrosis in salmonidae. Schweiz Arch. Tierheilkd., 116: 61-70 (In German).

Koroliuk, M.A., Ivanova, L.I., Majorova, I.G. and Tokarev, V.E. 1988. A method of determining catalase activity. Lab. Delo., 1: 16-19 (In Russian).

Kostiuk, V.A., Potapovich, A.I. and Kovaleva, Z.V. 1990. A simple and sensitive method of determination of superoxide dismutase activity based on the reaction of quercetin oxidation. Vopr. Med. Khim., 36: 88-91 (In Russian).

Kozińska, A. and Pękala, A. 2012. Characteristics of disease spectrum in relation to species, serogroups, and adhesion ability of motile *Aeromonads* in fish. Sci. World J., 2012; doi: 10.1100/2012/949358

Kurhalyuk, N. and Tkachenko, H. 2011. Induction of oxidative stress and antioxidant defenses in the liver of sea trout, *Salmo trutta* L., with ulcerative dermal necrosis. Arch. Pol. Fish., 19: 229-240. doi: 10.2478/v10086-011-0029-0

Kurhalyuk, N., Tkachenko, H. and Pałczyńska, K. 2009. Antioxidant enzymes profile in the brown trout (*Salmo trutta trutta*) with ulcerative dermal necrosis. Bull Vet Inst Pulawy, 53: 813-818.

Kurhalyuk, N., Tkachenko, H. and Pałczyńska, K. 2010. Lipid peroxidation and antioxidant defense system in spawn of brown trout (*Salmo trutta* m. *trutta* L.) affected by ulcerative dermal necrosis. Arch Pol Fish, 18: 115-122. doi: 10.2478/v10086-010-0013-0

Kurhalyuk, N., Tkachenko, H. and Pałczyńska, K. 2011. Resistance of erythrocytes from brown trout (*Salmo trutta* m. *trutta* L.) affected by ulcerative dermal necrosis syndrome. Pol J Vet Sciences, 14: 443-448. doi: 10.2478/v10181-011-0065-0

Law, M. 2001. Differential diagnosis of ulcerative lesions in fish. Environ. Health Perspect, 109: 681-686. doi: 10.2307/3454913

Levine, R.L., Garland, D., Oliver, C.N., Amici, A., Climent, I., Lenz, A.G., Ahn, B.W., Shaltiel, S. and Stadtman, E.R. 1990. Determination of carbonyl content in oxidatively modified proteins. Methods Enzymol., 186: 465-478. doi: 10.1016/0076-6879(90)86141-H

Limón-Pacheco, J. and Gonsebatt, M.E. 2009. The role of antioxidants and antioxidant-related enzymes in protective responses to environmentally induced oxidative stress. Mutat. Res, 674: 137-147.

Majumdar, T.S., Datta, D., Ghosh, S., Dutta, A., Chakraborty, R., Goswami, R. and Mazumder, S. 2007. Role of virulence plasmid of *Aeromonas hydrophila* in the pathogenesis of ulcerative disease syndrome in *Clarias batrachus*. Indian J. Biochem. Biophys, 44: 401-406.

McGarey, D.J., Milanesi, L., Foley, D.P., Reyes, B.J., Frye, L.C. and Lim, D.V. 1991. The role of motile aeromonads in the fish disease, ulcerative disease syndrome (UDS). Experientia, 47: 441-444.

Meier, W., Klingler, K. and Müller, R. 1977a. Ulcerative dermal necrosis (UDN) of the river trout (*Salmo trutta fario*) in Switzerland. II. Predisposing and infection-precipitating factors. Schweiz Arch Tierheilkd, 119: 277-291 (In German).

Meier, W., Klingler, K., Müller, R. and Luginbüh, H. 1977b. Ulcerative dermal necrosis (UDN) of the river trout (*Salmo trutta fario*) in Switzerland. I. Macroscopical and microscopical findings. Schweiz. Arch. Tierheilkd, 119: 235-245 (In German).

Moin, V.M. 1986. A simple and specific method for determining glutathione peroxidase activity in erythrocytes. Lab Delo, 12: 724-727 (In Russian).

Munro, A.L. 1970. Ulcerative dermal necrosis, a disease of migratory salmonid fishes in the rivers of the British Isles. Biol Conserv, 2: 129-132. doi: 10.1016/0006-3207(70)90148-5

Nakazawa, H., Genka, C. and Fujishima, M. 1996. Pathological aspects of active oxygens/free radicals. Jpn. J. Physiol., 46: 15-32. doi: 10.2170/jjphysiol.46.15

Nijveld, R.J., van Nood, E., van Hoorn, D.E.C., Boelens, P.G., van Norren, K. and van Leeuwen, P.A.M. 2001. Flavonoids: a review of probable mechanisms of action and potential applications. Am. J. Clin. Nutr., 74: 418-425.

Olivier, G.R., Lallier, R. and Larivière, S. 1981. A toxigenic profile of *Aeromonas hydrophila* and *Aeromonas sobria* isolated from fish. Can. J. Microbiol., 27(3): 330-333.

Pryor, W.A. 1986. Oxy-radicals and related species: their formation, lifetimes and reactions. Annu. Rev. Physiol., 48: 657-667. doi: 10.1146/annurev.physiol.48.1.657

Řehulka, J. 2002. *Aeromonas* causes severe skin lesions in

rainbow trout (*Oncorhynchus mykiss*): clinical pathology, haematology and biochemistry. Acta. Vet. Brno., 71: 351-360.

Roberts, R.J. 1993. Ulcerative dermal necrosis (UDN) in wild salmonids. Fish. Res., 17: 3-14. doi: 10.1016/0165-7836(93)90003-P

Roberts, R.J., Shearer, W.M., Munro, A.L. and Elson, K.G. 1969. The pathology of ulcerative dermal necrosis of Scottish salmon. J. Pathol., 97: 563-565. doi: 10.1002/path.1710970315

Sagara, Y., Dargusch, R., Chambers, D., Davis, J., Schubert, D. and Maher, P. 1998. Cellular mechanisms of resistance to chronic oxidative stress. Free Radic. Biol. Med., 24: 1375-1389. Doi: 10.1016/S0891-5849(97)00457-7

Sohal, R.S. 2002. Role of oxidative stress and protein oxidation in the aging process. Free Radic. Biol. Med., 33: 37-44.

Stadtman, E.R. and Levine, R.L. 2000. Protein oxidation. Ann. NY. Acad. Sci., 899: 191-208. doi: 10.1111/j.1749-6632.2000.tb06187.x

Szewczyk, E. 2005. Bacteriological diagnostic. Warszawa, PWN (In Polish).

Tauler, P., Gimeno, I., Aguilo, A., Guix, M.P., Pons, A., 1999. Regulation of erythrocyte antioxidant enzyme activities in athletes during competition and short-term recovery. Pflügers Archiv: European Journal of Physiology, 438: 782-787. doi: 10.1007/s004240051107

Tkachenko, H., Kurhalyuk, N. and Pałczyńska, K. 2011. Responses of antioxidant status in the gills of brown trout (*Salmo trutta* m. *trutta* L.). J. Ecol. Protec. Coastline, 15: 149-162.

Ursini, F. and Bindoli, A. 1987. The role of selenium peroxidases in the protection against oxidative damage of membranes. Chem. Phys. Lipids, 44: 255-276. doi: 10.1016/0009-3084(87)90053-3

Valavanidis, A., Vlahogianni, T., Dassenakis, M. and Scoullos, M. 2006. Molecular biomarkers of oxidative stress in aquatic organisms in relation to toxic environmental pollutants. Ecotoxicol. Environ. Saf., 64: 178-189. doi: 10.1016/j.ecoenv.2005.03.013

Ventura, M.T. and Grizzle, J.M. 1988. Lesions associated with natural and experimental infections of *Aeromonas hydrophila* in channel catfi sh, *Ictalurus punctatus* (Rafi nesque). Journal of Fish Diseases, 11: 397–407.

Wdzieczak, J., Zalesna, G., Wujec, E. and Peres, G. 1982. Comparative studies on superoxide dismutase, catalase and peroxidase levels in erythrocytes and livers of different freshwater and marine fish species. Comp Biochem Physiol, 73(B): 361-365. doi: 10.1016/0305-0491(82)90298-X

Yagi, K. 1993. Active oxygens, lipid peroxides, and antioxidants. Japan Scientific Societies Press, Japan.

Zar, J.H. 1999. Biostatistical Analysis. Prentice-Hall, Inc., Englewood Cliffs, New Jersey, 736 pp.