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OXIDATIVE STRESS RESPONSE IN THE CARDIAC TISSUE OF BALTIC SEA TROUT (*SALMO TRUTTA* M. *TRUTTA* L.) AFFECTED BY *AEROMONAS* SPP. INFECTION

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Abstract

Furunculosis induced by motile aeromonads is a problem in farming of salmonids (brown and rainbow trout) and various other fish species in the Europe during last years. Motile aeromonads cause diverse pathological conditions that include acute, chronic and covert infections (Cipriano and Austin 2011). Severity of disease is influenced by a number of interrelated factors, including bacterial virulence, the kind and degree of stress exerted on a population of fish, the physiological condition of the host and the degree of genetic resistance inherent within specific populations (Cipriano and Austin 2011). Numerous studies support the contribution of reactive oxygen species in the pathogenesis of parasite invasion mechanisms, as well as activation of immune system (Paiva and Bozza 2014). However, the validation of oxidative stress-related biomarkers in these settings is still lacking and novel association of these biomarkers and other biomarkers such as antioxidant defenses, is just emerging. Oxidative stress has been suggested as a pathogenic factor and therapeutic target in infective mechanisms. The aim of the present study was to examine the responses of oxidative stress biomarkers in the cardiac tissue of healthy specimens of sea trout (*Salmo trutta* m. *trutta* L.) and naturally furunculosis-affected trout in Słupia, the river in basin of the Baltic Sea where trout are spawned (northern Poland, Central Pomeranian region). The activities of antioxidant enzymes (superoxide dismutase, catalase, glutathione reductase, glutathione peroxidase, total antioxidant capacity), as well as oxidative stress biomarkers (2-thiobarbituric acid reactive substances as lipid peroxidation biomarker, aldehydic and ketonic derivatives as protein damage biomarker), and correlation between those in the cardiac tissue of healthy and furunculosis-affected trout were assayed. Furunculosis induces the production of aldehydic and ketonic derivatives of protein oxidation both in males and females. High protein oxidation occurred together with an alteration of the antioxidant defenses. Oxidative stress in cardiac tissue occurs more significantly in males of furunculosis-affected sea trout and contributes to the oxidative effect. It is probable that the cardiac cells which survive to the reactive oxygen species (ROS) have increased glutathione-dependent enzymes activity. However, this activation of the antioxidant system may not be sufficient to neutralize all the ROS, which does not allow an efficient cellular adaptation to

this furunculosis-induced stress. It also explains the lower total antioxidant capacity described among males and females when compared with healthy trout. These results may be explained by the decrease of superoxide dismutase activity, which may be caused by the furunculosis-induced ROS generation. A better understanding of the bacterium-induced oxidative stress in sea trout is required to find a cures.

Key words: sea trout (*Salmo trutta* m. *trutta* L.), furunculosis, *Aeromonas hydrophila*, oxidative stress, antioxidant defenses, Pomeranian region

INTRODUCTION

The motile species, *Aeromonas hydrophila*, has worldwide distribution, is among the most common bacteria in freshwater habitats and it frequently causes disease in cultured and feral fish (Austin and Austin 2007). Motile aeromonads are often referred to as a complex of disease organisms that are associated with bacterial haemorrhagic septicaemias and other ulcerative conditions in fish (Cipriano and Austin 2011). Notwithstanding, motile aeromonads are ubiquitous in most freshwater environments and are common in the water column and in the upper layers of sediment (Hazen 1979). They are adapted to environments that have a wide range of conductivity, turbidity, pH, salinity and temperature (Hazen et al. 1978). Temperature optima may depend on the particular strain, but generally range from 25 to 35°C (Cipriano and Austin 2011).

Resistance to disease in frogs and warmwater fish is lowered in the spring because aquatic organisms are often anaemic and have a substantial decrease in serum proteins resulting from periods of dormancy and starvation that have occurred during the winter (Cipriano and Austin 2011). Also, Huizinga et al. (1979) indicated that rising water temperatures increased metabolism, decreased overall condition and stressed the fish. These animals increased production of corticosteroids, which in turn increased their susceptibility to infection (Huizinga et al. 1979, Cipriano and Austin 2011).

A. hydrophila characteristically induced necrosis of muscle bundles, septic haemorrhages, lesions in the spleen, fatty livers, atrophy of renal haematopoietic tissue and necrosis in nephrons (Chien and Chieh 1994). 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. However, splenic ellipsoids are often centres of intense phagocytic activity by macrophages. Bach et al. (1978) observed pathological changes in the spleens of fish injected with virulent *A. hydrophila*, whereas fish infected orally showed little or no splenic involvement. Bacteria were present within the reticular sheaths of the ellipsoids, where intense phagocytic activity by macrophages occurred. Phagocytized bacteria divided intracellularly and extracellularly and destroyed the endothelial and reticular cells of the ellipsoids. The lower intestine and

vent, which sometimes protrude from the body, are often swollen, inflamed and haemorrhagic. Additionally, the intestine is devoid of food and may be filled with a yellow mucus-like material (Cipriano and Austin 2011).
Chronic motile aeromonad infections manifest themselves primarily as ulcerous forms of disease, in which dermal lesions with focal haemorrhages and inflammation are apparent. Both the dermis and epidermis are eroded and the underlying musculature becomes severely necrotic (Huizinga et al. 1979). Inflammatory cells are usually lacking in the necrotic musculature, whereas the adjacent epidermis undergoes a hyperplasia that results in a raised margin. At this stage, the infection has usually become systemic and petechial haemorrhages may occur throughout the peritoneum and musculature (Cipriano and Austin 2011).
Fish with only cutaneous infections may have several types of concealed lesions, including increased amounts of lipofuscin and haemosiderin in the liver and spleen; however, most visceral organs were not necrotic (Ventura and Grizzle 1988). Hepatic necrosis has been observed in channel catfish with systemic, cutaneous and latent infections caused by *A. hydrophila*, whereas hepatic steatosis was only evident in systemic infections (Grizzle and Kiryu 1993). In these same studies, pancreatic atrophy and necrosis were also evident in systemically diseased fish. Infiltration of leucocytes in necrotic areas of the liver and intrahepatic exocrine pancreas of infected fish was not observed, nor was there any correlation between infection and hepatic haemorrhage or haemosiderosis (Cipriano and Austin 2011).
Research in fish metabolism and parasite invasion mechanisms has advanced the knowledge of the mechanisms whereby parasites evade or cope with fish immune response. However, the validation of oxidative stress-related biomarkers in these settings is still lacking and novel association of these biomarkers and other biomarkers such as antioxidant defenses, is just emerging. Oxidative stress has been suggested as a pathogenic factor and therapeutic target in infective mechanisms (Paiva and Bozza 2014). A better understanding of the bacterium-induced oxidative stress in sea trout is required to find a cures. Therefore, the aim of the present study was to examine the responses of oxidative stress biomarkers in the cardiac tissue of healthy specimens of sea trout (*Salmo trutta* m. *trutta* L.) and naturally furunculosis-affected trout in Słupia, the river in basin of the Baltic Sea where trout are spawned (northern Poland, Central Pomeranian region). The activities of antioxidant enzymes (superoxide dismutase, catalase, glutathione reductase, glutathione peroxidase, total antioxidant capacity), as well as oxidative stress biomarkers (2-thiobarbituric acid reactive substances as lipid peroxidation biomarker, aldehydic and ketonic derivatives as protein damage biomarker), and correlation between those in the cardiac tissue of healthy and furunculosis-affected trout were assayed.

MATERIALS AND METHODS

Fish and Sampling. 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 close co-operation from the Landscape Park "The valley of Słupia" as well as the Board of Polish Angling Society in Słupsk. The sampling for analysis

was consisted from healthy males (n = 34) and females (n = 63) (control group), as well as males (n = 57) and females (n = 36) furunculosis-affected sea trout (study group) (Fig. 1). Tissues were sampled directly after catch. After catching, microbiological tests were carried out. These tests confirmed that *Aeromonas hydrophila* complex caused furunculosis (Szewczyk 2005). The cultivation of samples on the Aeromonas Isolation Agar for detecting of *Aeromonas spp.* suggested that *Aeromonas hydrophila complex* caused ulcerative dermal necrosis. The pathogen was isolated from the infected brown 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 (Daubner 1967) and the 0.2 mL of suspension was inoculated onto 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, using the following antimicrobial agents: chlorphenicol (30 μg), erythromycin (15 μg), gentamicyn (10 μg), methicillin (5 μg), nalidixic acid (30 μg), neomycin (30 μg), novobiocin (30 μg), rifampicin (5 μg), tetracycline (30 μg), and vancomycin (30 μg).

Fig. 1. Sea trout infected by *Aeromonas* spp.

Tissue homogenate preparation. Specimens in each group were dissected. One fish was used for each preparation. Each sample was homogenized in cold Tris-HCl buffer (100 mM, pH 7.2) to obtain a 10% (w/v) tissue homogenate. The homogenates were then centrifuged at 5,000 g for 15 min. Each supernatant was collected and stored at -25°C until use. The protein content of each sample was determined using Bradford method (1976) and bovine serum albumin as the standard.

Chemicals. Thiobarbituric acid (TBA), oxidized and reduced glutathione (GSSG and GSH), $NADPH_2$, 5,5-dithiobis-2-nitrobensoic acid (DTNB) were purchased from Sigma (St. Louis, MO, USA). Ethylenediaminetetraacetic acid (EDTA), thrichloroacetic acid (TCA), quercetin, hydrogen peroxide (H_2O_2), tetramethylenediamine (TEMED), ammonium molybdate, sodium aside, t-butylhydroperoxide, Tween 80, urea, 2,4-dinitrophenyl hydrazine (DNFH), were obtained from Fluka (Buchs, Switzerland). All other chemicals were of analytical grade.

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.

2-Thiobarbituric acid reactive substances (TBARS) assay for lipid peroxidation. Lipid peroxidation was determined by quantifying the concentration of 2-thiobarbituric acid reactive substances (TBARS) according to Kamyshnikov (2004). Briefly, a 2.1 mL subsample of homogenate was mixed with 1 mL of TCA and 1 mL of TBA reagent. The mixture was heated in a boiling water bath for 10 minutes. After cooling, the mixture was centrifuged at 3,000 g for 10 minutes. The TBARS level was expressed in nmol MDA per mg protein by using $1.56 \cdot 10^5 mM^{-1} \cdot cm^{-1}$ 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 0.1 M DNPH (dissolved in 2 M HCl) was added to 0.1 ml of the tissue samples after the protein was denatured. After adding the DNPH solution (or 2 M 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 (aldehydic derivatives, OMP_{370}) and 430 nm (ketonic derivatives, 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). Briefly, 1.0 mL of C reagent was mixed with 0.1 mL of tissue homogenate (1:1000). C reagent was made *ex tempore* (mixture of equal volumes of 0.1 M K,Na-phosphate buffer, pH = 7.8 and 0.08 M EDTA), pH of C reagent was adjusted to 10.0 by adding TEMED. Distilled water (0.1 mL) was added to blank vials instead of tissue 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 tissue 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. 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 tissue homogenate was measured according to the method described by Glatzle et al. (1974). 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 tissue homogenate. The rate of $NADPH^+$ oxidation was followed spectrophotometrically at 340 nm. A blank without $NADPH^+$ was used. 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 tissue homogenate was measured spectrophotometrically as described by Moin (1986). The assay mixture contained 0.8 mL of 0.1 M Tris-HCl with 6 mM EDTA and 12 mM sodium aside (pH 8.9), 0.1 mL of 4.8 mM GSH, 0.2 mL of tissue homogenate, 1 mL of 20 mM t-butylhydroperoxide, and 0.1 mL of 0.01 M DTNB. 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 sample was estimated spectrophotometrically at 532 nm following the method with Tween 80 oxidation (Halaktionova et al. 1998). Briefly, 0.2 mL of tissue homogenate was added to 2 mL 1% Tween 80. Blank assay instead of sample included 0.1 mL of distilled water. The mixture was heated in boiling water bath for 48 hours at 37°C. After cooling, 1 mL of TCA was added, and 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 that of the blank. TAC was expressed in %.

Statistical analysis. Data were presented as the mean ±S.E.M. and were checked for assumptions of normality using the Kolmogorov-Smirnov one-sample test and Lilliefors tests ($p > 0.05$). In order to find significant differences (significance level, $p < 0.05$) between healthy and furunculosis-affected groups, Mann-Whitney *U* test was applied to the data (Zar 1999). In addition, the relationships between oxidative stress biomarkers of all individuals were evaluated using Spearman's correlation analysis. All statistical analysis was performed by STATISTICA 10.0 software (StatSoft, Poland).

RESULTS

The values of lipid peroxidation in the cardiac tissue of the males and females from control (healthy specimens) and furunculosis-affected trout are summarized in Fig. 2. No significant differences between healthy and furunculosis-affected males were observed, while TBARS level in cardiac tissue of females was non-significant lower (by 50%, $p > 0.05$) compared to healthy trout (Fig. 2).

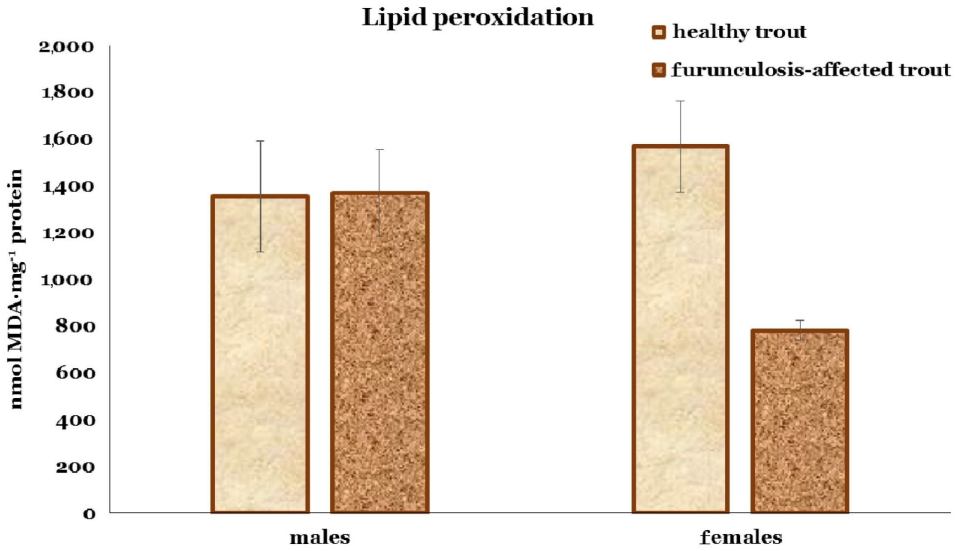


Fig. 2. The TBARS level (nmol MDA per mg protein) in the cardiac tissue of the furunculosis-affected sea trout, which returning to spawning from the Baltic Sea
Data are represented as mean ±S.E.M.

Protein carbonylation is the most commonly used measure of oxidative modification of proteins (Stadtman and Levine 2003, Wehr and Levine 2013). Protein carbonyls formed on several amino acids residues, including arginine, histidine, lysine, proline, threonine and cysteine, are the most widely used biomarker for measurement of protein oxidation and oxidative stress in diseases (Yan 2009, Wehr and Levine 2013, Cai and Yan 2013). As the modification occurs on multiple amino acid residues on selected protein targets, its magnitude is much greater than any other modifications that occur only on a specific amino acid residue, and thus is more readily detectable (Yan 2009, Cai and Yan 2013). Aldehydic derivatives of oxidatively modified proteins in the cardiac tissue of furunculosis-affected males and females were significantly increased (by 99%, $p = 0.037$ and by 65%, $p = 0.000$, respectively) compared to healthy trout (Fig. 3). Furunculosis caused a significant increase the amount of ketonic derivatives in the cardiac tissue of males by 63.5% ($p = 0.043$) and females by 48.7% ($p = 0.000$) compared to healthy trout. Higher level of ketonic derivatives of oxidatively modified protein was noted in cardiac tissue both in healthy females

(by 26%, p = 0.017) and furunculosis-affected females (by 14.5%, p = 0.007) compared to respectively male groups (Fig. 3).

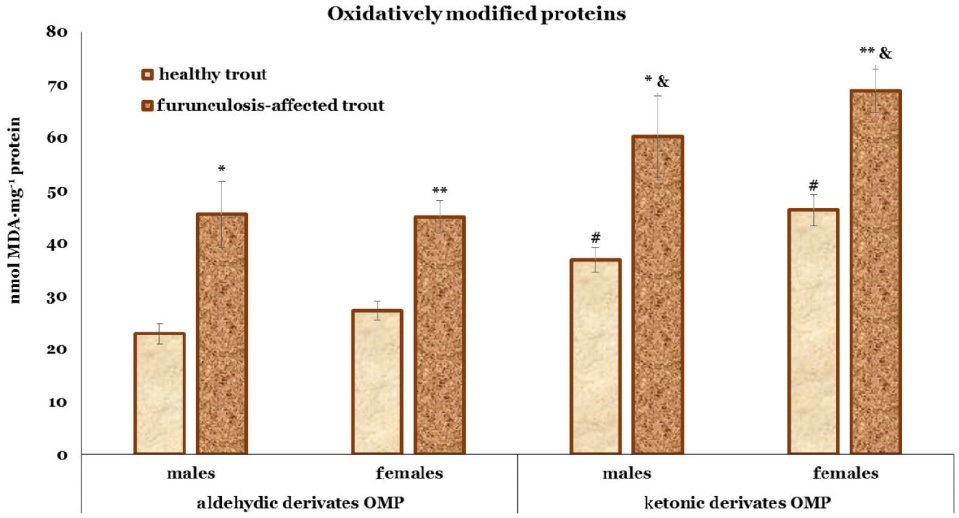


Fig. 3. Aldehydic and ketonic derivatives of oxidatively modified proteins in the cardiac tissue of the furunculosis-affected sea trout, which returning to spawning from the Baltic Sea
Data are represented as mean ±S.E.M.

* the significant difference was shown as $p < 0.05$ when compared healthy and furunculosis-affected males

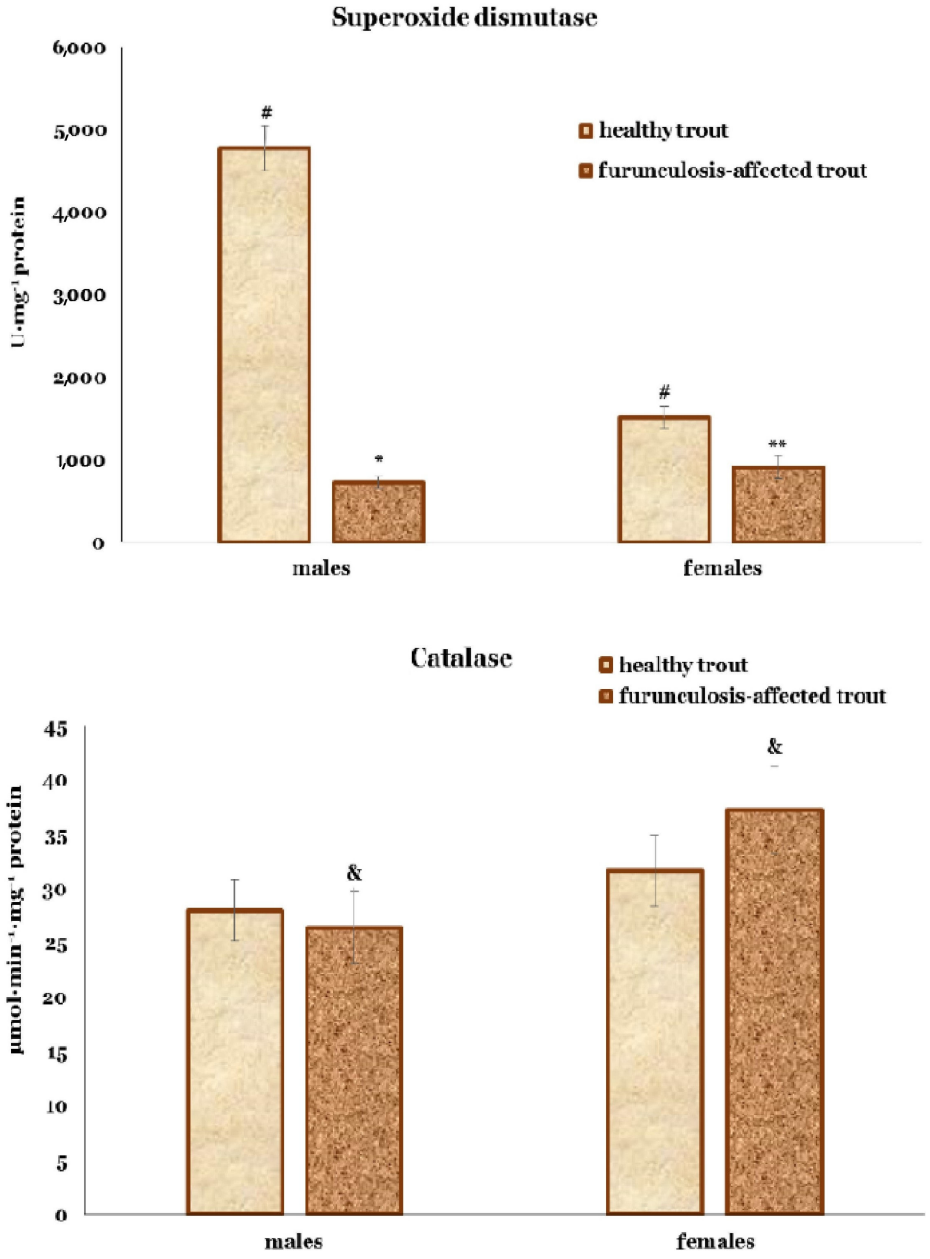
** the significant difference was shown as $p < 0.05$ when compared healthy and furunculosis-affected females

the significant difference was shown as $p < 0.05$ when compared healthy males and healthy females

& the significant difference was shown as $p < 0.05$ when compared furunculosis-affected males and furunculosis-affected females

A wide array of non-enzymatic and enzymatic antioxidant defenses exists, act systematically to scavenge the free radicals (Matés and Sánchez-Jiménez 1999). The enzymatic antioxidant system consists of superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), and glutathione reductase (GR). This system is the main defense system against ROS *in vivo*. There are two major types of SOD. One is Cu,Zn-SOD (SOD1), which mainly exist in cytoplasm, with copper and zinc being present in the active site. The other one is Mn-SOD (SOD2), locating in mitochondrial matrix, with manganese being present in the active site. They can catalyze the reaction to decompose superoxide anion radicals into H_2O_2, which will then be converted to water and oxygen by CAT or GPx. CAT is one of the most efficient redox enzymes, with iron being present in its active site, mainly found in peroxisome. It can catalyze the conversion of H_2O_2 into water and oxygen. Otherwise, H_2O_2 would be converted to hydroxyl radical, one of the most active and harmful radicals to living cells. GPx is a selenium-containing enzyme, protecting cells and tissues from oxidative damage by removing H_2O_2 with the oxidization of glutathione. On

the other hand, GR can convert the oxidized glutathione to its reduced form (Peng et al. 2014). Superoxide dismutase, catalase, glutathione reductase, and glutathione peroxidase activities in the cardiac tissue of the furunculosis-affected sea trout, which returning to spawning from the Baltic Sea are presented in Fig. 4.



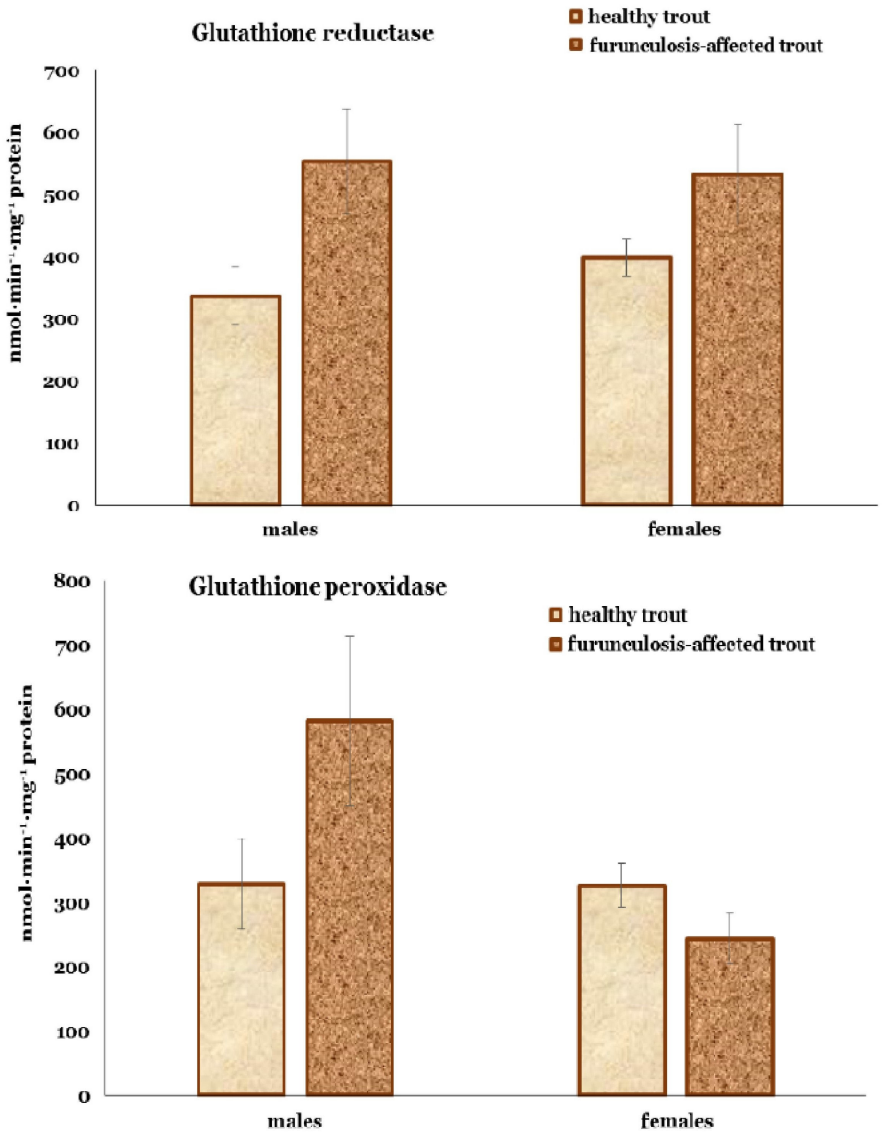


Fig. 4. Superoxide dismutase, catalase, glutathione reductase, and glutathione peroxidase activities in the cardiac tissue of the furunculosis-affected sea trout, which returning to spawning from the Baltic Sea.

Data are represented as mean ±S.E.M.

* the significant difference was shown as $p < 0.05$ when compared healthy and furunculosis-affected males

** the significant difference was shown as $p < 0.05$ when compared healthy and furunculosis-affected females

the significant difference was shown as $p < 0.05$ when compared healthy males and healthy females

& the significant difference was shown as $p < 0.05$ when compared furunculosis-affected males and furunculosis-affected females

SOD activity in cardiac tissue of furunculosis-affected sea trout was decreased by 85% ($p = 0.000$) in males and by 40% ($p = 0.001$) in females compared to healthy trout. Cardiac GR activity was non-significantly higher in furunculosis-affected sea trout compared to healthy fish. Non-significant changes in CAT and GPx activities in the cardiac tissue of the furunculosis-affected sea trout were observed (Fig. 4).
The total antioxidant capacity (TAC), which is another marker used for indirectly determining the levels of oxidative stress in tissue (Bisogni et al. 2012). TAC was significantly decreased in the cardiac tissue of the furunculosis-affected males compared to those in the healthy trout (by 47%, $p = 0.000$). Furunculosis-affected females have higher TAC level (by 64%, $p = 0.001$) compared to respective males group (Fig. 5).

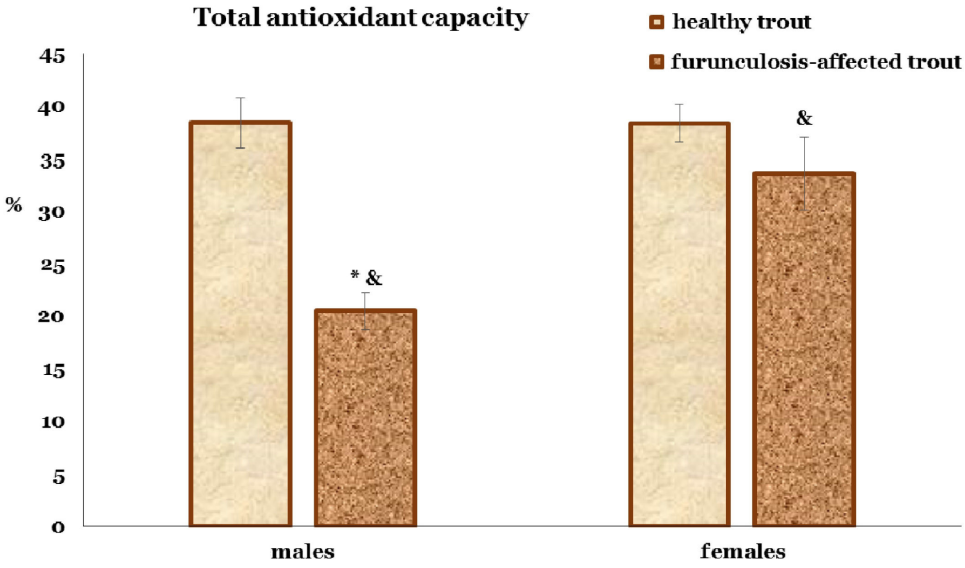


Fig. 5. Total antioxidant capacity (TAC, %) activities in the cardiac tissue of the furunculosis-affected sea trout, which returning to spawning from the Baltic Sea
Data are represented as mean ±S.E.M.

* the significant difference was shown as $p < 0.05$ when compared healthy and furunculosis-affected males

& the significant difference was shown as $p < 0.05$ when compared furunculosis-affected males and furunculosis-affected females

Correlations between oxidative stress markers in the cardiac tissue of the furunculosis-affected sea trout, which returning to spawning from the Baltic Sea are presented in Figs 6-9. Low level of aldehydic and ketonic derivatives of oxidatively modified proteins are supported by high total antioxidant capacity ($r = -0.635$, $p = 0.000$ and $r = -0.615$, $p = 0.000$, respectively). Moreover, the main role in total antioxidant capacity plays catalase ($r = -0.496$, $p = 0.003$) to maintain the lipid peroxida-

tion at low level (r = 0.400, p = 0.019) in the cardiac tissue of healthy males of sea trout (Fig. 6).

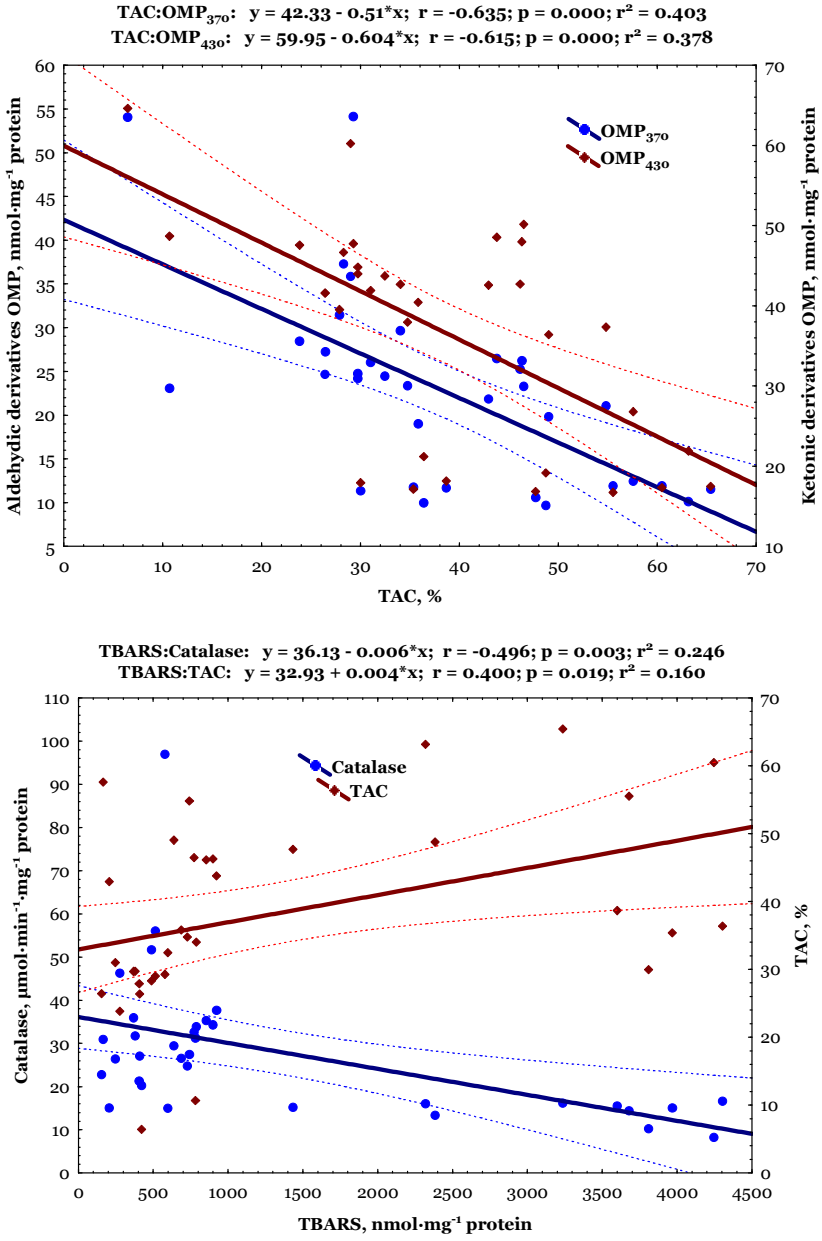


Fig. 6. Correlations between total antioxidant capacity, aldehydic and ketonic derivatives of oxidatively modified proteins content, as well as between TBARS level, catalase activity, and TAC in the cardiac tissue of healthy males of sea trout, which returning to spawn from the Baltic Sea

Generation of aldehydic derivatives of oxidatively modified proteins activates the GR activity (r=0.851, p=0.000) and decreases the total antioxidant capacity (r = -0.313, p = 0.018) in the cardiac tissue of furunculosis-affected males of sea trout (Fig. 7). Additionally, generation of ketonic derivatives of oxidatively modified proteins activates the GPx activity (r = -0.279, p =0.036) and also decreases the total antioxidant capacity (r = -0.313, p = 0.018) (Fig. 7).

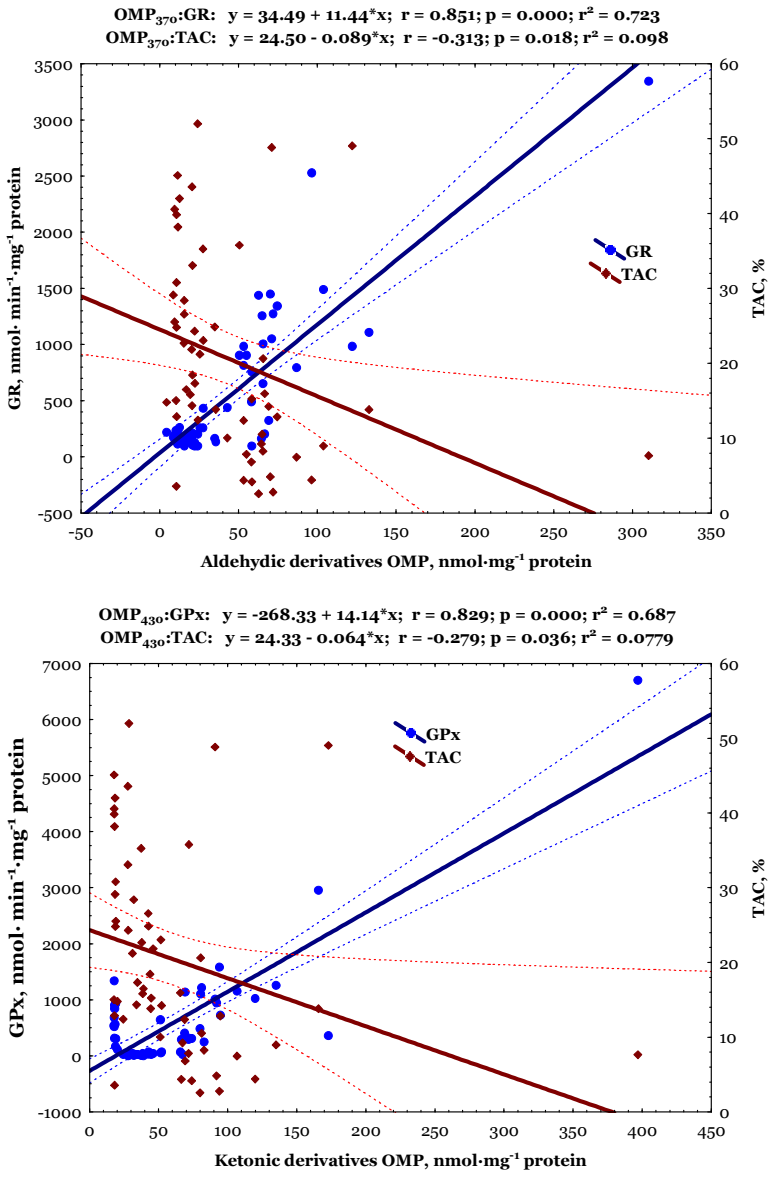


Fig. 7. Correlations between aldehydic and ketonic derivatives of oxidatively modified proteins, GR and GPx activity in the cardiac tissue of furunculosis-affected males of sea trout, which returning to spawning from the Baltic Sea

High activity of catalase (r = -0.466, p = 0.000) and GR activity (r = -0.536, p = 0.000) maintains a low level of lipid peroxidation. Moreover, catalase (r = 0.561, p = 0.000) and SOD activity (r = 0.414, p = 0.001) determine the accumulation of aldehydic derivatives of oxidatively modified protein in the cardiac tissue of healthy females of sea trout, which returning to spawning from the Baltic Sea (Fig. 8).

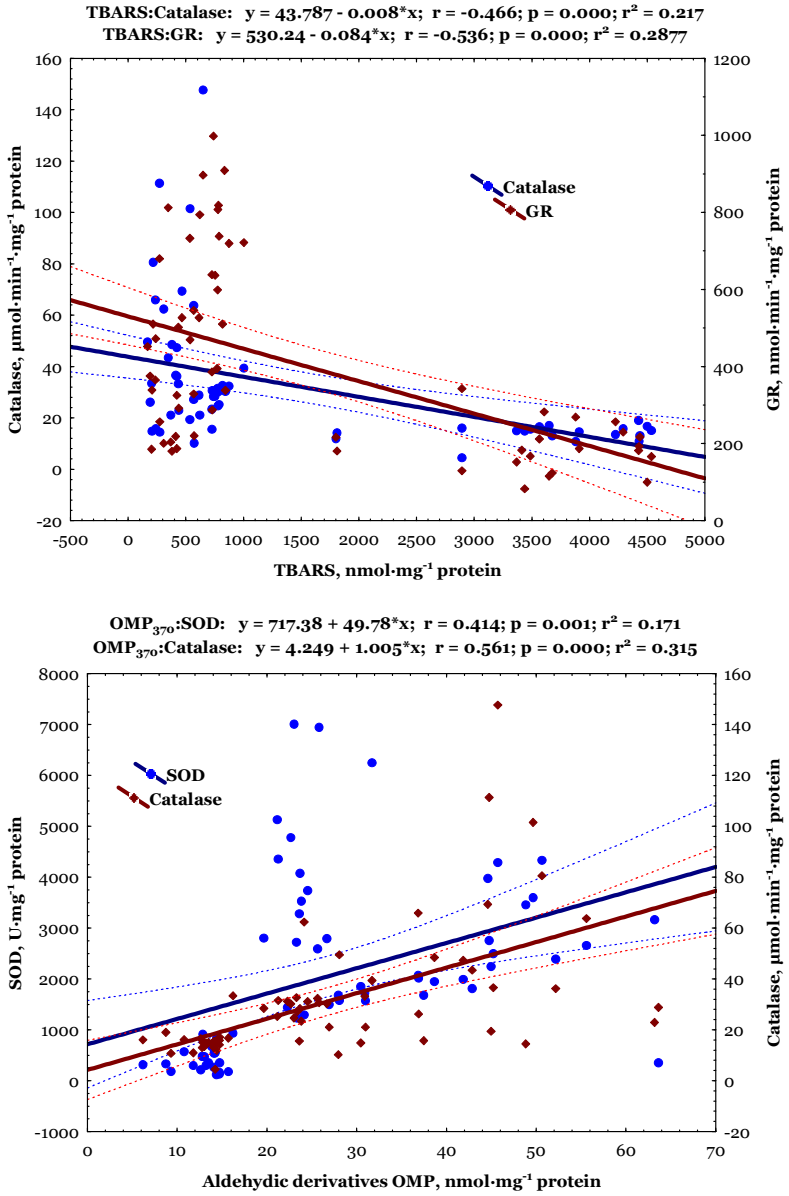


Fig. 8. Correlations between aldehydic derivatives of oxidatively modified proteins, TBARS level, SOD, catalase, and GR activity in the cardiac tissue of healthy females of sea trout, which returning to spawning from the Baltic Sea

In the cardiac tissue of furunculosis-affected females of sea trout, generation of aldehydic and derivatives of oxidatively modified proteins decreases the total antioxidant capacity (r = -0.490, p = 0.002 and r = -0.665, p = 0.000, respectively) and activates the GR (r = 0.525, p = 0.001) and GPx activity (r = 0.588, p = 0.000) (Fig. 9).

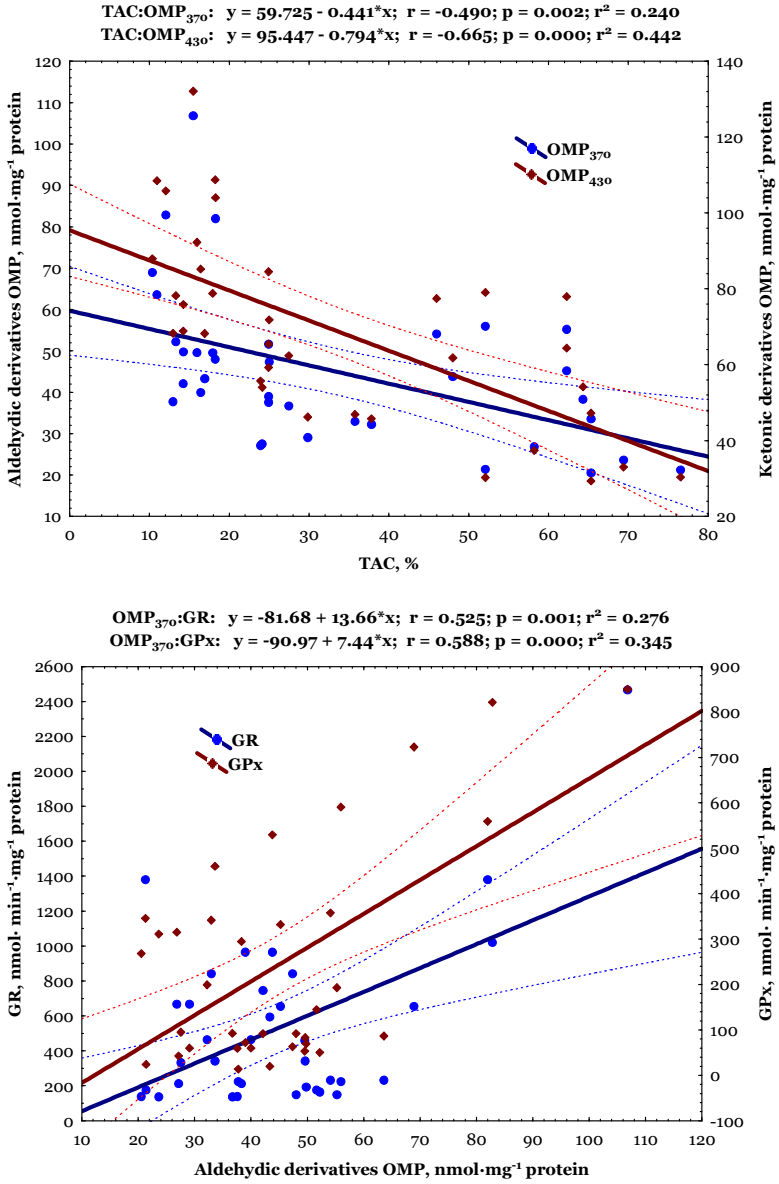


Fig. 9. Correlations between total antioxidant capacity, aldehydic and ketonic derivatives of oxidatively modified proteins, as well as between GR and GPx activity in the cardiac tissue of furunculosis-affected females of sea trout, which returning to spawning from the Baltic Sea

DISCUSSION

Furunculosis is a ubiquitous disease that affects aquaculture operations worldwide and is characterized by high mortality and morbidity (Dallaire-Dufresne et al. 2014). A better understanding of the bacterium-induced oxidative stress in sea trout is required to find a cure. Thereby, our study centers on oxidative stress and antioxidant defense responses in the cardiac tissue of sea trout which returning to spawning from the Baltic Sea. The lipid peroxidation, the oxidatively modified proteins, such as aldehydic and ketonic derivatives as well as the activities of superoxide dismutase, catalase, glutathione reductase, and glutathione peroxidase activities, total antioxidant capacity, and correlations between these indices are described (Figs 2-8).

Furunculosis induces respiratory burst as response to *Aeromonas* spp. infection (Kilpi et al. 2013). These authors sequentially challenged rainbow trout representing 15 full-sibling families twice with *A. salmonicida* causing furunculosis: first as cohabitation and then as injected intraperitoneally. The bleeding procedure prior to challenges caused the outbreak of cold water disease by *Flavobacterium psychrophilum*. Before and after the outbreak and challenges, 11 immunological parameters were measured from blood samples. The immunological responses predicted the fate of the fish since nearly all the initial responses were lower in individuals which later died from cold water disease than in survivors. Fish died from furunculosis had impaired respiratory burst response to *A. salmonicida*. The outbreak and challenges resulted in these individuals higher and faster responses compared with initial values. Unlike in mammals, the number of monocytes, but not that of granulocytes, in rainbow trout blood correlated well with the whole blood respiratory burst activity (Kilpi et al. 2013).

Results of Nikoskelainen et al. (2007) suggest that the monocytes play a more significant role than the granulocytes in antibody-dependent opsonophagocytosis during vaccination against *A. salmonicida*. Nikoskelainen et al. (2007) investigated the effects of vaccination and composition of vaccine on the plasma antibody levels, biological function of antibodies in opsonophagocytosis as well as the effects of vaccination on the blood leucocyte counts. Vaccine 1 contained *Aeromonas salmonicida*, *Listonella anguillarum* and both Th and Fd serotypes of *Flavobacterium psychrophilum* antigens and vaccine 2 contained *A. salmonicida*, *L. anguillarum* and only Fd serotype of *Fl. psychrophilum*. The antibody-mediated opsonophagocytosis was determined as the respiratory burst activity of blood monocytes and granulocytes against the tested bacterial antigens. Three weeks after vaccination both vaccine groups and the control group showed increased respiratory burst activity against all bacterial strains. However, the increase in respiratory burst activities was non-specific and originated from the increased number of circulating granulocytes and monocytes. On the other hand, at 6 weeks post-vaccination both specific antibodies and antibody-dependent opsonophagocytosis appeared in both vaccine groups. However, the composition of the vaccine had a marked effect on the magnitude of specific responses. Both polyvalent vaccines appeared to mainly affect the numbers of circulating monocytes (Nikoskelainen et al. 2007).

In this study, we have provided evidence that furunculosis induces the production of free radicals in the cardiac tissue of sea trout. Also, the role of ROS in furunculosis-

affected trout is proposed. Pathogen invasion leads to oxidative burst and generation of active oxygen species, causing lipid peroxidation, oxidatively modification of proteins and membrane damage, which are believed to be key features of pathogenesis (Paiva and Bozza 2014). In the present study, protein oxidation mediated membrane damage was evident (Fig. 3).

High levels of aldehydic and ketonic derivatives of proteins oxidation were observed both in males and females of sea trout (Fig. 3). The assays showed that high protein oxidation occurred together with an alteration of the antioxidant defenses (decrease of SOD activity). The data indicated that these two events occur simultaneously. Therefore, we decided to conduct the correlative analysis between oxidative stress and antioxidant defense biomarkers. In furunculosis-affected males, generation of aldehydic derivatives of oxidatively modified proteins activates the GR activity ($r = 0.851$, $p = 0.000$) and decreases the total antioxidant capacity ($r = -0.313$, $p = 0.018$) (Fig. 7). Additionally, generation of ketonic derivatives of oxidatively modified proteins activates the GPx activity ($r = -0.279$, $p = 0.036$) and also decreases the total antioxidant capacity ($r = -0.313$, $p = 0.018$) (Fig. 7). In furunculosis-affected females, generation of aldehydic and derivatives of oxidatively modified proteins decreases the total antioxidant capacity ($r = -0.490$, $p = 0.002$ and $r = -0.665$, $p = 0.000$, respectively) and activates the GR ($r = 0.525$, $p = 0.001$) and GPx activity ($r = 0.588$, $p = 0.000$) (Fig. 9). Therefore, while there is considerable evidence linking ROS accumulation to antioxidant defenses, there are a confirm the intrinsic relationship between ROS accumulation and GR and GPx activities occurrence and their regulation.

Oxidation of proteins can lead to hydroxylation of aromatic groups and aliphatic amino acid side chains, nitration of aromatic amino acid residues, nitrosylation of sulfhydryl groups, sulfoxidation of methionine residues, chlorination of aromatic groups and primary amino groups, and to conversion of some amino acid residues to carbonyl derivatives. Oxidation can lead also to cleavage of the polypeptide chain and to formation of cross-linked protein aggregates. Furthermore, functional groups of proteins can react with oxidation products of polyunsaturated fatty acids and with carbohydrate derivatives (glycation/glycoxidation) to produce inactive derivatives (Stadtman and Levine 2003). The results show that furunculosis infection of sea trout resulted in an increase in the amount of aldehydic and ketonic derivatives of OMP, while lipid peroxidation was non-changed (Figs 2 and 3). Based on these data, it was hypothesized that furunculosis does not have a unique target in the cardiac cells: infection induces the production of ROS, leading to protein oxidation, which corresponds to the oxidative degradation of proteins, in which a free radical chain 'steals' electrons from the amino acids in cell membranes, resulting in cell disfunction. This phenomenon would happen in a dynamic way together with inhibiting antioxidant defenses. In addition, furunculosis was able to induce the decrease of total antioxidant capacity in cardiac tissue of sea trout (Fig. 4).

We observed no significant changes of lipid peroxidation in cardiac tissue of furunculosis-affected sea trout; on the other hand, it was possible to note an increase in OMP level and SOD activity, as well as GR and GPx activities at this time. Synergism between glutathione-dependent enzymes activity and aldehydic and ketonic derivatives of oxidatively modified proteins was also verified. These results suggest that oxidative stress in cardiac tissue occurs more significantly in males of furuncu-

losis-affected sea trout and contributes to the oxidative effect. It is probable that the cardiac cells which survive to the ROS have increased antioxidant activity. This hypothesis helps to explain the fact that activities of glutathione-dependent enzymes were increased in cardiac tissue of males and females of furunculosis-affected sea trout (Fig. 4). Moreover, these results complete the previous our studies reporting an inhibition of antioxidant defense system and activation of oxidative stress biomarkers in the blood, gills, liver, spawn, and muscles of sea trout with furunculosis (Kurhalyuk et al. 2009, 2010, 2011, Kurhalyuk and Tkachenko 2011, Tkachenko et al. 2011, 2014a, b).

Glutathione-mediated antioxidant defense system appears to be important in protecting cells against furunculosis-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 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 (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). SOD, acting as the first line of defense against ROS, converts superoxide to H_2O_2. There are essentially three types of SODs containing manganese, iron, or copper plus zinc as prosthetic metals (Peng et al. 2014). The resultant H_2O_2 can be detoxified to oxygen and water by CAT (Peng et al. 2014).

The assays demonstrated non-significant increased GR and GPx activity, with synergism between glutathione-dependent enzymes and oxidative stress biomarkers. However, this activation of the antioxidant system may not be sufficient to neutralize all the ROS, which does not allow an efficient cellular adaptation to this furunculosis-induced stress (Figs 4, 5, 7, 9). It also explains the lower TAC described among males and females when compared with healthy trout (Fig. 5). These results may be explained by the reduction of SOD activity, which may be caused by the ROS generation.

CONCLUSIONS

1. Furunculosis infection of sea trout resulted in an increase in the amount of aldehydic and ketonic derivatives of OMP, while lipid peroxidation was non-changed. Based on these data, it was hypothesized that furunculosis does not have a unique target in the cardiac cells: infection induces the production of reactive oxygen species, leading to protein oxidation, which corresponds to the oxidative degradation of proteins, in which a free radical chain 'steals' electrons from the amino acids in cell membranes, resulting in cell disfunction. This phenomenon would happen in a dynamic way together with inhibiting antioxidant defenses. In addition,

furunculosis was able to induce the decrease of total antioxidant capacity in cardiac tissue of sea trout.

2. Activities of glutathione-dependent enzymes were increased in cardiac tissue of males and females of furunculosis-affected sea trout. Synergism between glutathione-dependent enzymes activity and aldehydic and ketonic derivatives of oxidatively modified proteins suggest that oxidative stress in cardiac tissue occurs more significantly in males of furunculosis-affected sea trout and contributes to the oxidative effect. However, this activation of the antioxidant defenses may not be sufficient to neutralize all the reactive oxygen species, which does not allow an efficient cellular adaptation to this furunculosis-induced stress.
3. Low level of aldehydic and ketonic derivatives of oxidatively modified proteins are supported by high total antioxidant capacity in the cardiac tissue of healthy males. Moreover, the main role in total antioxidant capacity plays catalase to maintain the lipid peroxidation at low level. In healthy females, high activity of catalase and GR activity maintains a low level of lipid peroxidation. Moreover, catalase and SOD activity determine the accumulation of aldehydic derivatives of oxidatively modified protein. Generation of aldehydic derivatives of oxidatively modified proteins activates the GR activity and decreases the total antioxidant capacity in the cardiac tissue of furunculosis-affected males of sea trout. Additionally, generation of ketonic derivatives of oxidatively modified proteins activates the GPx activity (and also decreases the total antioxidant capacity. In furunculosis-affected females, generation of aldehydic and derivatives of oxidatively modified proteins decreases the total antioxidant capacity and activates the GR and GPx activity.
4. Finally, our data showed an interesting role of oxidative stress caused by furunculosis in the cardiac tissue of males and females of sea trout. We believe that these findings are important to a better understanding of the mechanism of furunculosis infection in trout with follow understanding of mechanisms in treatment procedure against *Aeromonas*-caused infections.

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STRES OKSYDACYJNY W TKANCE SERCA BAŁTYCKIEJ TROCI WĘDROWNEJ (*SALMO TRUTTA* M. *TRUTTA* L.) ZAINFEKOWANEJ *AEROMONAS* SPP.

Streszczenie

Furunkuloza indukowana przez ruchliwych *Aeromonas* była problemem w hodowli ryb łososiowatych (pstrąg tęczowy i pstrąg potokowy) i wielu innych gatunków ryb w Europie w ubiegłych latach. Ruchliwe *Aeromonas* mogą wywoływać różne stany patologiczne, które obejmują ostre, przewlekłe i ukryte stany infekcyjne (Cipriano i Austin 2011). Ciężkość choroby zależy od wielu czynników, m.in. wirulencji bakterii, rodzaju i stopnia czynników stresowych działających na populację ryb, stanu fizjologicznego i stopnia odporności genetycznej w określonych populacjach (Cipriano i Austin 2011). Liczne badania potwierdzają udział reaktywnych form tlenu (RFT) w patogenetycznych mechanizmach inwazji pasożyta, a także w aktywacji układu immunologicznego (Paiva i Bozza 2014). Jednak wpływ stresu oksydacyjnego w tych procesach jest nadal niewyjaśniony przy ocenie kondycji fizjologicznej zainfekowanych ryb. Stres oksydacyjny jest czynnikiem patogennym i terapeutycznym w mechanizmach patologicznych procesów zakaźnych. Celem niniejszej pracy było zbadanie poziomu biomarkerów stresu oksydacyjnego w tkance serca u zdrowych i naturalnie zainfekowanych osobników troci wędrownej (*Salmo trutta* m. *trutta* L.) w Słupi, rzece w basenie Morza Bałtyckiego (północna Polska, Pomorze środkowe), gdzie troć odbywa swoje tarło. Aktywność enzymów antyoksydacyjnych (dysmutaza ponadtlenkowa, katalaza, reduktaza glutationowa, peroksydaza glutationowa, całkowita aktywność antyoksydacyjna) oraz stężenie markerów stresu oksydacyjnego (produkty reagujące z kwasem 2-tiobarbiturowym jako biomarkery peroksydacji lipidów, aldehydowe i ketonowe pochodne jako biomarkery oksydacyjnej modyfikacji białek) oznaczono w tkance sercowej zdrowych i naturalnie zainfekowanych osobników troci wędrownej. Jak potwierdzają wyniki naszych badań, furunkuloza indukuje wytwarzanie aldehydowych i ketonowych pochodnych oksydacyjnej modyfikacji białek ze zmianą aktywności enzymów obrony antyoksydacyjnej zarówno u samców, jak i samic troci. Stres oksydacyjny w tkance mięśnia sercowego występuje w większym stopniu u zainfekowanych samców. Tkanka sercowa naturalnie zainfekowanych osobników troci wędrownej ma zwiększoną aktywność enzymów zależnych od glutationu. Jednakże aktywacja układu antyoksydacyjnego całkowicie nie ogranicza stresu oksydacyjnego w tkance sercowej zainfekowanych ryb, co powoduje zmniejszenie całkowitej aktywności antyoksydacyjnej.