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ANTIOXIDANT ENZYMES PROFILE IN THE BROWN TROUT (*SALMO TRUTTA TRUTTA*) WITH ULCERATIVE DERMAL NECROSIS

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Abstract

Eighty individuals of migratory brown trout *(Salmo trutta trutta)* from the River Słupia (Central Pomerania) during spawning were studied. The liver, muscles, and heart from 13 healthy males and 26 healthy females (control group) as well as from 23 males and 18 females with ulcerative dermal necrosis (UDN; study group) were collected directly after catch of the fish. Our investigations showed that UDN coincides with the functioning of antioxidative system defences. The pathophysiological mechanism of UDN impact is connected with a decrease in the most important antioxidant enzymes (superoxide dismutase, catalase, glutathione peroxidase, and glutathione reductase) and total antioxidant activity of muscles, liver, and heart. Increases in oxidative stress markers and intensity of lipid peroxidation level caused by UDN are suggested.

Key words: brown trout, ulcerative dermal necrosis, lipid peroxidation, oxidative stress, Poland.

Ulcerative dermal necrosis (UDN) is a serious fish disease, which has been spreading across Poland since in the last few years (2). The disease was first noted in Poland in 1923-1924 in the River Dunajec. The disease spread very rapidly in 2007-2008, affecting from 8.3% (River Słupia) to 58.3% (River Rega) of the brown trout (*Salmo trutta trutta*) in the Pomeranian rivers (2). Since then, the disease has recurred annually, affecting a variety of fish species in a number of rivers in Northern Poland. The brown trout is one of the food fish in Poland. This species is found in both freshwater and brackish water habitats in Baltic Sea, and is suitable for aquaculture. The most severely affected fish frequently die before spawning.

Affected fish develop severe skin lesions over large parts of their body, which penetrate into muscles (4, 5, 7, 11, 17). The onset of symptoms occur only after migration into freshwater. Lesions become quickly infected with overgrowths of *Saprolegnia* fungus, giving the affected fish an appearance of being covered in slimy white pustules (14, 22). The initial signs of the disease are circles of pathologically-changed epidermis. Subsequently, the intercellular spaces dilate and communicate with the exterior. Necrosis of the epidermal cells occurs simultaneously with fungal infections and marked responses of the melanophores (19, 20). The aetiology of the outbreaks of UDN remains unknown.

There has been no conclusive evidence of the involvement of any particular organism as the primary pathogen (22). It is suggested that the fungal infections are triggered by metabolites of the necrotic epidermal cells (14). The epidermal cells are shed and the fungus determines the further course of the disease, which terminates in large ulcers covered with fungal hyphae (14, 19, 20).

Environmental pollution, temperature changing, hormonal stress under spawning, change in food support, manipulating stress under fish cultivation, and damage to epidermal cells cause oxidative stress, aggravated by UDN (1, 22).

So far, only a few studies have been conducted on the pathology of UDN in brown trout populations (5, 11, 19, 20). In the present study, the main goal was to investigate the effects of UDN-induced oxidative stress on parameters of lipid peroxidation and activities of antioxidative defence system *in vivo* systems. Selected pro- and ant-ioxidative profile parameters of the liver, muscle tissue, and heart of healthy brown trout were studied and compared with those of UDN-positive fish from the River Słupia to evaluate the effects, if any, of UDN on the general antioxidative enzymes activities and lipid peroxidation of this species.

Material and Methods

Experimental groups. The experiments were carried out during 2008. The specimens of brown trout, 3-5 years of age, were collected from 13 healthy males and 26 healthy females (control group) as well as from 23 males and 18 females with ulcerative dermal necrosis (UDN; study group), just after fish-catching in the River Słupia (Nothern Poland, Central Pomerania). The fishing took place with the thorough co-operation with the Landscape Park "The valley of Słupia" as well as with the Board of Polish Angling Relationship in Słupsk. Only UDN-positive fish with bodies covered by more than 3-5 white pustules and with skin changes affecting more than 10%-15% of its skin surface, were investigated.

Individuals from both groups were transported to the laboratory in cages with local water and, after necropsy, sections of the liver, muscles, and heart were collected and analysed within a day after of the sampling procedure. Each procedure of tissue analysis was carried out at a temperature of around 2-4^{o}C.

Chemicals. 2-Tiobarbituric acid (TBA), reduced glutathione (GSH), oxidised glutathione (GSSG), serum albumin, NADPH, 5,5-dithio-bis(2-nitrobenzoic acid) (DTNB), and quercetine were purchased from Sigma. All chemicals were of analytical grade.

Biochemical assays. The tissues were homogenised in 0.1 M tris-HCl buffer (pH 7.4). The protein content was determined according to Bradford (3) using bovine serum albumin as a standard.

Superoxide dismutase (E.C. 1.15.1.1**).** Superoxide dismutase (SOD) activity was measured using quercetine oxidation as a substrate after suitable dilution following the method by Kostyuk *et al*. (16). The assay mixture in a total volume of 1 ml consisted of 0.08 mM of EDTA and 0.1 M of sodium phosphate buffer (pH 7.8) in the proportion of 1:1. Briefly, 0.1 ml of homogenate (diluted 1:1,000) was added to 2.3 ml of distilled water, followed by the addition of 1 ml of assay mixture with EDTA and a sodium phosphate buffer. The activity was calculated as the difference between a decrease in quercetine oxidation lasting 20 min after adding to assay mixture. The decrease in absorbance was recorded at 406 nm, using spectrophotometer (SPECOL SP 1103 LanOptics). In the blank, homogenate was substituted by an equal quantity of distilled water. SOD activity was expressed as units mg^{-1} of soluble protein, one unit of SOD activity being defined as the amount of protein causing 50% inhibition of the rate of quercetine oxidation.

Catalase (E.C. 1.11.1.6). Catalase (CAT) activity was estimated by measuring the breakdown of hydrogen peroxide in the reaction mixture using a spectrophotometer at the wave length of 410 nm by the method of Korolyuk *et al.* (15). The reaction was initiated by adding 0.1 ml of the homogenate to 2 ml of 0.03% H_2O_2 solution and 1 ml of 4% ammonium molibdate. One unit of catalase activity was defined as the amount of enzyme required to clear 1 μmol of H_20_2 per min. Results were related to the soluble protein.

Glutathione reductase (E.C. 1.6.4.2). Glutathione reductase (GR) activity was measured according to the method described by Glatzle *et al.* (8). The reaction enzyme assay mixture contained 2.4 ml of 0.067 M sodium phosphate buffer (pH 6.6), 0.2 ml of 7.5 mM oxidised glutathione, 0.2 ml of 6 mM NADPH, and an aliquot of homogenate (0.1 ml). The rate of NADPH oxidation was followed spectrophotometrically at 340 nm. In the blank, a reaction assay mixture without NADPH was used. The specific GR activity was expressed as nmol NADPH per min and referred to the soluble protein.

Glutathione peroxidase (E.C. 1.11.1.9). The activity of glutathione peroxidase (GPO) was measured spectrophotometrically as described by Moin (18). The assay mixture contained 0.8 ml of 0.1 M tris-HCl with 6 mM EDTA and 12 mM sodium azide (pH 8.9), 0.1 ml of 4.8 mM GSH, 0.2 ml of the 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. GPO activity was expressed as μmol GSH per min. Results were related to the soluble protein.

Total antioxidative activity. The level of total antioxidative activity (TAA) was estimated following the method with Tween-80 oxidation (9). 0.2 ml of the tissue homogenate was added to 2 ml of 1% Tween-80. The mixture was heated in a boiling-water bath for 48 h at 40^{o}C. The mixture was then added 1 ml of thrichloroacetic acid and centrifuged at 1,500 g for 15 min. After centrifugation, 2 ml of 0.25% thiobarbituric acid reagent and 2 ml of supernatant were mixed. The mixture was heated in a boiling-water bath for 15 min at 90^{o}C. The solutions were read spectrophotometrically at 532 nm. The total antioxidative activity of tissue was expressed as a percentage.

Marker of oxidative damage. Lipid peroxidation was determined by the production of TBA-reactive substances (TBARS) (12). Briefly, 2.1 ml of homogenate was added to 1 ml of thrichloracetic acid and 1 ml of 2-thiobarbituric acid reagent. The mixture was heated in a boiling-water bath for 10 min. After cooling, the mixture was centrifuged at 1,500 g for 10 min. The absorbance of the chromogen was determined at 532 nm. The concentration of TBARS calculated by using 1.56×10^{5} $M^{-1}cm^{-1}$ as extinction coefficient and lipid peroxide level in the tissues was expressed in μmol of TBARS per mg of protein.

Statistical analysis. Results are expressed as mean ±SD. The significance of differences in enzyme activity ($P<0.05$) was examined using a one-way ANOVA followed by a multiple-range test (significance level, $P<0.05$), Levene's test, and Tukey HSD test (test of reasonably important difference for a contrived best statistical fit). Correlations between TBARS level and enzyme activity in the tissue samples at the set significance level were determined by the regression method. Interactions were established by the Pearson test for linear correlation (25). All statistical calculation was performed on separate data from each individual with STATISTICA version 8.0.

Results

We measured the malonic dialdehyde concentration, an end product indicating the extent of lipid peroxidation in the liver, muscles, and heart of healthy brown trout and compared with those of UDN-positive fish. Fig. 1 shows the intensity of lipid peroxidation in these tissues.

The lipid peroxide level in tissues from fish of UDN-positive group was significantly higher than the corresponding control value. The levels of TBARS were unchanged in the liver (test F=1.78, P=0.158), although different patterns were observed in the white muscles (test F=11.25, P=0.0) and heart (test F=13.73, P=0.0) from specimens of both males and females with UDN. Lipid peroxidation increased by 112% (test HSD P=0.026) in muscle tissue, although no changes were observed in the liver and heart from males with UDN. Lipid peroxidation in the heart from females with UDN was increased by 85% (test HSD P=0.0) compared with control females. TBARS content in muscle tissues from females with UDN was augmented by 85% (test HSD P=0.0) and remained high in the liver.

No significant changes were observed in SOD activity in muscles (test F=1.68, P=0.179) and liver (test F=1.20, P=0.317) from trout with UDN (Tables 1-3). UDN caused a 51% decrease in SOD activity of male hearts (test HSD P= 0.020). Catalase activity in the control trout was higher in the liver (54 μM/min·mg protein) than in the other organs (Table 1).

In the liver, catalase activity (test F=5.49, P=0.0) was decreased by 43% (test HSD P=0.01) in males with UDN and remained low (27% lower than controls) in females with UDN (Table 1). The lowest activities of catalase (test F=7.82, P=0.0) were observed (Table 2) in males (31%, P<0.05) compared with the corresponding controls. No significant changes were observed in catalase activity from the hearts of both males and females with UDN (Table 3). On the other hand, UDN caused a 46% decrease in muscle catalase activity of females.

GPO activity (test F=20.32, P=0.0) was decreased by 61% in the liver (P=0.0) of females with UDN (from 109.1 to 43 μM/min·mg protein). No significant changes in the activity of GPO were observed in the liver of males. The activity of GPO remained 25% lower than in male controls (Table 1). High control GPO activity was observed in the muscles of both males and females (334 and 323 μM/min·mg protein, respectively). UDN caused a decrease in GPO activity by 61% in the liver and by 41% in heart of females. GPO activity decreased by 53% and 58%, respectively in muscle tissue in both males and females with UDN compared with controls. The activity of the control GR was higher in the heart (426 and 182 nmol/min·mg protein, respectively) than in the other organs (Table 4). GR activity in muscles was very low in both male and female trout (33 and 48 nmol/min·mg protein, respectively). No changes in GR activity were observed in the liver from males with UDN. Liver GR activity in females decreased by 48% compared with controls. The activity of GR in muscles and heart from trouts with UDN remained within control levels.

The measurements of TAA in muscle tissue (test F=66.18, P=0.0), heart (test F=12.10, P=0.0), and liver (F=3.79, P=0.01) from trouts with UDN are shown in Fig. 2. A high level of control TAA was observed in the muscle of males and females (90.8% and 90.7%, respectively). UDN caused a decrease in TAA level by 46% and 61%, respectively. TAA level remained unchanged in the heart and liver of males and decreased in the heart and liver of females (by 47% and 31%, respectively) compared with controls. Data of correlative analysis of antioxidative parameters in the tissues of brown trout with UDN are shown in Table 4.

Correlative analysis suggest high correlative links between antioxidative parameters in the tissues of brown trout with UDN, especially TBARS level and SOD activity for males, and catalase activity and glutathione defence system for control males.

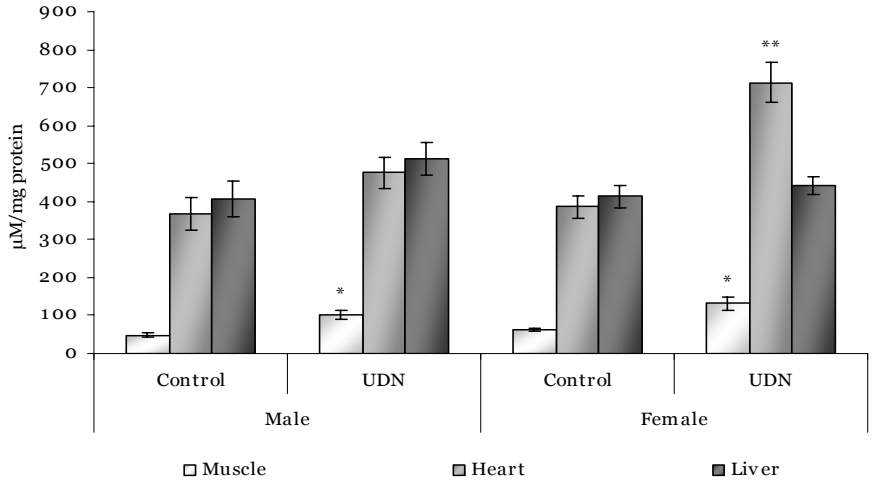

Fig. 1. The intensity of lipid peroxidation (TBARS level) in muscle tissues, heart, and liver of migratory brown trout (*Salmo trutta trutta*) with ulcerative dermal necrosis (UDN).

* difference is significant between control males and males with UDN (P<0.05),
** difference is significant between control females and females with UDN (P<0.05).

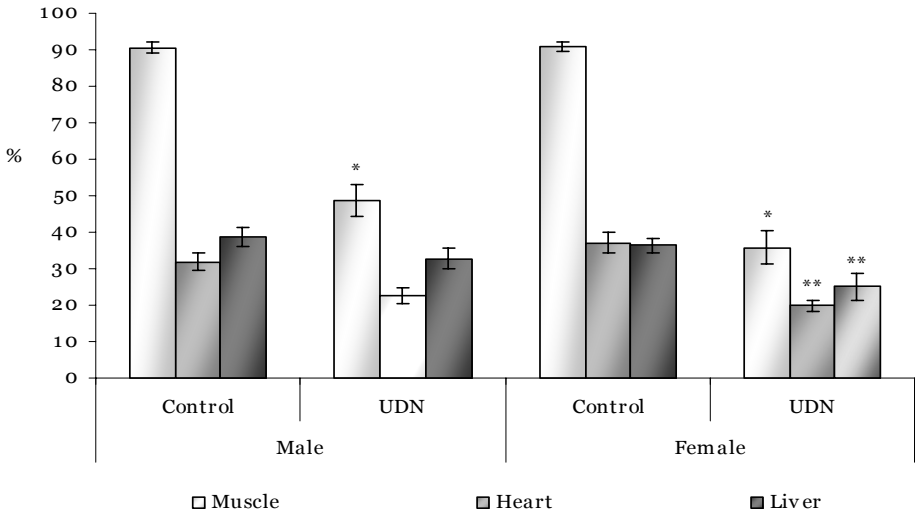

Fig. 2. The total antioxidative activity in muscle tissues, heart, and liver of brown trout (*Salmo trutta trutta*) with ulcerative dermal necrosis (UDN).

* difference is significant between the control males and males with UDN (P<0.05),
** difference is significant between the control females and females with UDN (P<0.05).

Table 1
The antioxidant enzyme activity in the liver of brown trout (*Salmo trutta trutta*) with ulcerative dermal necrosis (UDN)

Antioxidative enzyme activity	Males		Females	
	Control	UDN	Control	UDN
SOD	161.56±24.37	116.48±15.09 P=0.981	270.36±18.78	207.08±13.39 P=0.615
CAT	53.84±7.32	30.85±2.01 P=0.014	43.68±3.85	32.00±4.74 P=0.254
GR	73.69±8.74	52.92±3.84 P=0.335	98.48±8.51	51.05±5.11 P=0.0
GPO	83.32±7.04	62.67±9.19 P=0.604	109.09±11.15	43.02±4.31 P=0.0

Table 2
The antioxidant enzyme activity in muscle tissues of brown trout (*Salmo trutta trutta*) with ulcerative dermal necrosis (UDN)

Antioxidative enzyme activity	Males		Females	
	Control	UDN	Control	UDN
SOD	691.4±52.02	571.26±59.56 P=0.458	618.75±30.71	532.3±38.55 P=0.608
CAT	49.26±4.36	33.81±4.43 P=0.096	40.36±3.23	21.64±2.35 P=0.007
GR	32.80±5.04	40.07±4.32 P=0.879	47.59±6.52	42.25±4.39 P=0.917
GPO	334.10±21.98	155.52±15.11 P=0.0	323.08±30.47	134.16±9.13 P=0.0

Table 3
The antioxidant enzyme activity in the heart of brown trout (*Salmo trutta trutta*) with ulcerative dermal necrosis (UDN)

Antioxidant enzyme activity	Males		Females	
	Control	UDN	Control	UDN
SOD	184.72±17.47	94.89±10.76 P=0.020	231.82±18.67	172.32±19.35 P=0.101
CAT	36.71±6.18	24.44±3.23 P=0.546	47.08±6.6	34.28±2.84 P=0.367
GR	182.24±25.39	176.89±17.11 P=0.999	425.85±42.03	391.1±62.64 P=0.943
GPO	71.45±7.07	34.07±4.45 P=0.163	117.62±13.76	69.38±7.14 P=0.011

Table 4
A correlative analysis of antioxidative parameters in tissues from specimens of brown trout (*Salmo trutta trutta*) with ulcerative dermal necrosis (UDN)

Relation	Correlative coefficient, *r*	Regressive curve	Determinative coefficient, R^2	Significant difference level, P
Liver				
Males, SOD-GR	0.61	y=-622.1+30.37x	0.37	0.028
Males, CAT-TBARS UDN	0.52	y=154.42+11.6x	0.27	0.011
Females, CAT-TBARS UDN	0.50	y=361.26+2.49x	0.25	0.034
Females, GPO-CAT UDN	0.57	y=26.55+0.51x	0.32	0.014
Females, TBARS-GR UDN	- 0.63	y=11.53-0.137x	0.40	0.005
Muscles				
Males, SOD-TBARS	0.58	y=7.58+0.006x	0.33	0.039
Males, CAT-GPO	0.88	y=116.15+4.42x	0.77	0.000
Males, TBARS-GPO UDN	0.51	y=85.09+0.69x	0.26	0.013
Males, TBARS-TAA UDN	-0.56	y=71.46-0.22x	0.31	0.005
Males, GPO- TAA UDN	-0.66	y=79.19-0.2x	0.44	0.001
Heart				
Males, SOD-CAT	0.85	y=-18.63+0.03x	0.72	0.000
Males, SOD-TBARS	0.62	y=96.82+0.15x	0.38	0.025
Males, SOD-TAA	-0.64	y=48.66-0.009x	0.41	0.019
Females, GR-CAT	0.41	y=19.5+0.065x	0.17	0.036
Males, SOD-TBARS UDN	0.81	y=185.16+0.31x	0.66	0.000
Males, CAT-TBARS UDN	0.66	y=273.7+8.31x	0.44	0.001
Females, SOD-CAT UDN	0.66	y=17.65+0.0097x	0.43	0.003

Discussion

In the present study, the physiological response of UDN-affected brown trout was investigated. Specifically, we addressed the question of whether oxidative stress occurs during UDN and whether antioxidative defense system is induced. The interplay between these defence systems is important for the passive survival of oxidative stress caused by UDN. Enzymatic antioxidant defence are key components of in the biochemical machinery that allows the survival of species during oxidative stress (10, 13). To our knowledge, no study has yet been carried out on the problem of the potential oxidative stress in fish with UDN.

Among the organs analysed, the muscle tissue is the most subjected to oxidative stress in brown trout affected by UDN. Lipid peroxidation, quantified as the TBARS level, dramatically increased in muscles from both males and females, and in female heart (Fig. 1). This suggests that the overproduction of reactive oxygen species is a major problem in muscles and heart, and lipid peroxidative processes can be devastating to muscle-cell integrity in these cases. The fact that there was no change in liver lipid peroxidation of brown trout affected by UDN suggests that enzymatic decomposition of lipid peroxidative products is negligible in the liver. To search for the biochemical adaptations to this oxidative stress, we quantified the hepatic levels of TAA and the activity of four antioxidative enzymes.

We observed that the activity of catalase was significantly decreased in the liver of male trout affected by UDN (Table 1). The maintenance of high constitutive levels of other antioxidative enzyme activity (SOD, GPO, GR) may have controlled the extent of oxyradical-induced lipid peroxidation to a level that is physiologically tolerable for the organ (6, 24). Hydroxyl radicals are thought to be involved in the initiation and propagation of lipid peroxidation (13). GR and GPO are important in minimising the accumulation of the toxic products of lipid peroxidation, including malodialdehyde and lipid hydroperoxides (21, 23, 24). The decrease in GR and GPO activity caused a significant decrease in TAA level in the liver of female trout affected by UDN. Other antioxidant enzymes (SOD and catalase) stayed at high levels in the liver of females and low levels of hepatic lipid peroxidation in persisted female trout.

In the heart of females with UDN, GPO activity and TAA level were significantly decreased and TBARS level remained high (Fig. 1 and Table 3). Moreover, CAT and GR activities in the heart of male remained at high levels compared with other tissues. These results might explain why lipid peroxidation did not increase in the heart of male trout with UDN (Fig. 1).

In the case of muscles, most antioxidant defences remained at control levels. Exceptions were the decrease in muscle GPO activity and the TAA level in both male and female trout with UDN (Fig. 3 and Tables 2). Moreover, muscles had the lowest levels of GR activity among the tissues examined, and GPO activity and TAA level also significantly decreased. It is noteworthy that an organ with such low levels of antioxidant defences could decrease lipid peroxidation (Fig. 1). The levels of other antioxidant defensive compounds remained constant in muscles of trout with UDN. The

intensification of lipid peroxidation in muscle tissues and heart caused by UDN could be attributed to decreased antioxidant defence (23).

The investigations suggest increases in oxidative stress markers and intensity of lipid peroxidation level caused by UDN for both males and females. The pathophysiological mechanism of the impact of UDN is connected with a decrease in the most important antioxidant enzymes (catalase, glutathione peroxidase and glutathione reductase) and total antioxidant activity from muscles, liver and heart.

Correlative analysis of antioxidative parameters suggests strong relative links between lipid peroxidation and antioxidative enzyme activity in the tissues of brown trout with UDN. Oxidative damage may be minimised by antioxidant defence mechanisms that protect the cell against cellular oxidant and repair systems, which prevent the accumulation of oxidative-damaged molecules (10, 13). Any change in the antioxidative parameters in the metabolic pathways of fish reflected by environmental stress causes significant ecophysiological responses (23).

Our investigations show that the negative results of UDN coincide with the functioning of antioxidative defence system. In conclusion, UDN caused significant reductions in total antioxidative activity, the glutathione defence system, and a significant increase in the lipid peroxidation in the muscle tissues and heart of females. Decreases in catalase activity in the liver create unfavourable conditions for the elimination of free radicals, especially in males. Increases in oxidative stress markers and intensity of lipid peroxidation level caused by UDN have been suggested. More biochemical studies on other fish species are needed to characterise the pro- and antioxidative changes associated with UDN.

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