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Oxidative Stress and Antioxidant Enzyme Defence System in Seminal Plasma of Common Carp (*Cyprinus carpio*) and Rainbow Trout (*Oncorhynchus mykiss*) during Spawning Season

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ABSTRACT

Shaliutina-Kolešová A., Rui N., Ashtiani S., Rodina M., Cosson J., Linhart O. (2018): **Oxidative stress and antioxidant enzyme defence system in seminal plasma of common carp (*Cyprinus carpio*) and rainbow trout (*Oncorhynchus mykiss*) during spawning season**. Czech J. Anim. Sci., 63, 78–84.

Assessment of seminal oxidative stress indicators is emerging as a valuable prognostic tool in assisted reproductive technology. We investigated levels of oxidative stress caused by reactive oxygen species (ROS) and the antioxidant enzyme defence system comprising superoxide dismutase (SOD), glutathione reductase (GR), and glutathione peroxidase (GPx) in seminal plasma (SP) of common carp *Cyprinus carpio* and rainbow trout *Oncorhynchus mykiss* over the course of the spawning season. Oxidation was determined in lipids and proteins by assessing thiobarbituric acid reactive species (TBARS) and the 2,4-dinitrophenylhydrazine carbonyl groups, respectively. Levels of SOD were assessed by the autoxidation of pyrogallol. Determination of GR and GPx was based on the rate of NADPH oxidation. We observed clear alterations in lipids and proteins over the course of the spawning season in both species. The highest levels of TBARS and CP were recorded late in the season. SOD was not significantly altered in either species. The activity of GR was higher in carp SP late in spawning compared to other times ($P < 0.05$), while in rainbow trout, GR significantly increased (24.13 ± 2.8 mU/mg protein) in mid-season. A significantly lower GPx activity (9.18 ± 1.32 mU/mg protein) was found in rainbow trout SP early in the spawning season, but no significant differences in GPx were observed over the course of the season in carp. These results provide further understanding of the role of fish SP antioxidants and present new data on the oxidant and antioxidant balance in SP during the spawning season that may be of value in the development of methods for artificial reproduction of teleost species.

Keywords: reproductive season; lipid peroxidation products; fish sperm; superoxide dismutase; glutathione reductase; glutathione peroxidase

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The effects of seminal plasma (SP) composition on mature spermatozoa have been widely studied in several species with contradictory results. It has been demonstrated that SP is important for (1) activation and augmentation of spermatozoon motility (Bozkurt et al. 2011); (2) buffering to provide the optimal osmotic and nutrient medium; (3) prevention of premature activation during physiological transport of spermatozoa and stabilization of the plasma membrane with capacitation inhibitors (Villemure et al. 2003); (4) assistance in spermatozoon–ovum interactions (Souza et al. 2008); and (5) protection of spermatozoa from oxidative stress. In contrast, detrimental effects of SP on spermatozoon motility, viability (Garcia and Graham 1987), and survival after freeze/thaw (Schmehl et al. 1986) have also been reported. These data characterize SP as a complex combination of components impacting sperm survival and motility.

Season is an important factor influencing semen quality and fertilization capacity (Shaliutina-Kolesova et al. 2016). Studies have shown that spermatozoon motility parameters change during the spawning season in common carp *Cyprinus carpio*, tilapia *Oreochromis mossambicus* (Kruger et al. 1984), rainbow trout *Onchorhynchus mykiss* (Munkittrick and Moccia 1987), and common barbel *Barbus barbus* (Alavi et al. 2008). A significant variation in the protein composition of SP over the course of the spawning season has also been reported (Shaliutina-Kolesova et al. 2016). Seasonal changes in the SP protein content are likely due to the alterations in the protective effects of SP against cold-shock, possibly related to antioxidant enzyme activity (Marti et al. 2007).

SP contains enzymatic and non-enzymatic components (Lahnsteiner et al. 2010). Enzymatic antioxidant defence mechanisms include the glutathione peroxidase (GPx)/reductase (GR) system, superoxide dismutase (SOD), and catalase (Aitken 1999). The enzymes act as antioxidants and inhibitors of lipid peroxidation. Oxidative stress is a state related to increased cell damage triggered by oxygen and oxygen-derived free radicals known as reactive oxygen species (ROS). Due to the high levels of polyunsaturated fatty acids in plasma membranes, spermatozoa of both mammals (Lenzi et al. 2002) and fish (Pustowka et al. 2000) are particularly susceptible to oxidative damage. Imbalance between ROS and sperm antioxidant activity has been reported to be a primary cause of spermatozoon damage (Li et al. 2010). The generation of ROS is considered a major source of cryo-damage, especially of DNA (Thomson et al. 2009). Despite these findings, there is no available information on seasonal variation in the oxidant content and antioxidant activity of SP in fish.

The goal of the present study was to investigate seasonal variations in the indices of oxidative stress (thiobarbituric acid reactive substances and carbonyl derivatives of proteins) and activity of the SP antioxidant defence system comprising SOD, GR, and GPx in the distantly related fish species common carp *Cyprinus carpio* and rainbow trout *Oncorhynchus mykiss*.

Common carp and rainbow trout were selected, as they are among the most widely cultured fish species and constitute representative models for teleost studies.

MATERIAL AND METHODS

Ethics. All experiments were conducted according to the principles of the Ethics Committee for the Protection of Animals in Research of the University of South Bohemia in České Budějovice, Research Institute of Fish Culture and Hydrobiology, Vodňany, based on the EU-harmonized Animal Welfare Act of the Czech Republic.

Reagents. All media components were produced by Sigma-Aldrich (USA).

Experimental fish and rearing conditions. Sixteen male *Cyprinus carpio* (3 years, 3.5–4.0 kg) and fifteen male *Oncorhynchus mykiss* (3 years, 3.0 kg) were reared at the experimental station of the Faculty of Fisheries and Protection of Waters at Vodňany, University of South Bohemia in České Budějovice (Czech Republic). Rainbow trout and carp were maintained separately in 10 000 l outdoor hatchery tanks with constant flowing pond water. Water temperature followed natural seasonal variation and ranged from 5 to 8°C, pH was 6.0–8.5, and dissolved oxygen concentration 8–9 mg O_2/l during the rainbow trout spawning season, and from 20 to 22°C, pH 7.0–7.5, and 6.5–7.1 mg O_2/l in the carp spawning season. Natural light entered the tanks via netting which prevented fish from escaping and provided variable light intensity according to the external environmental conditions and time of day. A solution of malachite

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green (2 mg/ml, zinc free; Sigma-Aldrich) was occasionally used to prevent the spread of fungal infections. Fish were fed three times weekly, but unfed for 24–48 h before manipulation.

Semen collection and experimental design. Fish were maintained under the natural photoperiod, and sperm was obtained during the natural reproduction season in both species. No hormone treatment was used. Sperm of rainbow trout was collected at three time points in the reproductive season: early (10 March 2014), middle (24 March 2014), and late (2 April 2014). Carp were stripped on 21 May 2014, 20 June 2014, and 23 July 2014, corresponding to early, middle, and late time points in the reproductive season.

Sperm samples were collected into 5 ml syringes by abdominal massage. We attempted to collect all milt at each stripping and took care to avoid contamination with urine, mucus, or blood. Oxygen was provided to the sperm by maintaining sufficient headspace in the syringe. Syringes were placed on ice (4°C) and immediately transported to the laboratory for analyses.

The bicinchoninic acid assay, using the photometer Infinite M200 (Tecan, Switzerland) for reading, was employed to determine protein concentrations in the samples.

Lipid peroxidation products. The levels of thiobarbituric acid reactive substances (TBARS) and carbonyl derivatives of proteins (CP) were measured as an index of oxidative stress resulting from lipid peroxidation. Briefly, sperm samples were centrifuged at 13 000 *g* at 4°C for 10 min. The supernatant was collected and suspended in 50 mM potassium phosphate (KPi) buffer, pH 7.0, containing 0.5 mM EDTA and homogenized in an ice bath using a Sonopuls HD 2070 ultrasonicator (Bandelin Electronic, Germany). The homogenate was divided into two portions, one for measuring TBARS and CP levels, and the other was centrifuged at 12 000 *g* for 30 min at 4°C to obtain the post-mitochondrial supernatant for the investigation of antioxidant enzyme activity. The TBARS method (Li et al. 2010) was used to evaluate lipid peroxidation (LPO) in fish SP. The concentration of TBARS was calculated by absorption at 535 nm and a molar extinction coefficient of 156 mM/cm and expressed as nmol per mg of protein. Carbonyl derivatives of proteins were detected by reaction with 2,4-dinitrophenylhydrazine (DNPH) as described by Lenz et al. (1989). The quantity of CP was measured spectrophotometrically at 370 nm using a molar extinction coefficient of 22 mM/cm and expressed as nmol per mg of protein. Oxidative stress was calculated in triplicate for each sample.

Antioxidant parameters evaluation

Evaluation of superoxide dismutase activity in seminal fluid. SOD activity was determined by the method of Marklund and Marklund (1974) based on the autoxidation of pyrogallol. The prepared SP samples were centrifuged at 12 000 *g* at 4°C for 30 min. The SOD activity was assessed spectrophotometrically at 420 nm and expressed as the quantity of enzyme per mg of protein. One unit of SOD activity is defined as the amount of the enzyme necessary to produce 50% dismutation of the superoxide radical per min. Results were expressed as international milliunits (mU) per mg of protein and obtained in triplicate for each sample.

Evaluation of glutathione reductase and glutathione peroxidase activity in seminal fluid. GR activity was determined spectrophotometrically, measuring NADPH oxidation at 340 nm. GPx activity was assayed, based on the rate of NADPH oxidation at 340 nm, by the coupled reaction with GR. Specific activity was determined using the extinction coefficient of 6.22 mM/cm (Lawrence and Burk 1976). One unit of GPx or GR activity was defined as the quantity of the enzyme that consumes 1 μmol of substrate or generates 1 μmol of product per min. The activity was expressed as mU/mg protein. GPx and GR activities were assessed in triplicate for each sample.

Data analysis. Normality and the homogeneity of variance of all data were tested with the Kolmogorov test and the Bartlett test, respectively. Because multiple samples were taken from the same individual, repeat measures ANOVA followed by Tukey's post hoc test, which accounts for the season effect as well as the between-fish variation, were applied to evaluate seasonal changes in TBARS and CP levels and antioxidant activity in SP. Data were expressed as means ± standard deviation (carp n = 16 and rainbow trout n = 15). Analyses were performed at the significance level of 0.05 using STATISTICA software Version 9.0 for MS Windows.

RESULTS AND DISCUSSION

Changes in sperm quality during the spawning season have been reported in teleost fish. Spermatozoon

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motility is used as an indicator of sperm quality. In our previously published study (Shaliutina-Kolesova et al. 2016), we found the highest motility rates and velocity at 5 s post-activation in the middle of the spawning season in carp and rainbow trout. The percentage of motile spermatozoa decreased late in the spawning period. This led to speculation that oxidative stress can be an important factor contributing to reduced spermatozoon motility late in the spawning season.

ROS are generally produced as a by-product of normal aerobic metabolism and have an important impact on sperm physiology (Bhattacharjee 2010). A balance in ROS levels is apparently crucial, since an inappropriate production rate leads to infertility (Pasqualotto et al. 2001). An excess of ROS production initiates peroxidative lipid damage and interferes with spermatozoon motility, apparently through the depletion of adenosine triphosphate (ATP), which is independent of the inhibition of oxidative phosphorylation (Gazo et al. 2015). SP exhibits substantial antioxidant capacity. The major role of antioxidants is in the inactivation or transformation of oxidants into less reactive forms or reaction with antioxidant molecules that are chemically stable.

Lipid peroxidation has been reported to be a major contributor to the loss of cell function under oxidative stress and has usually been indicated by TBARS level in fish (İnanan et al. 2016). In the present study, the TBARS level varied from 0.51 ± 0.11 to 1.05 ± 0.15 nmol/mg protein in carp, and no significant differences were observed between samples from early and mid-spawning season. In rainbow trout, the maximum level of TBARS in SP (0.95 ± 0.05 nmol/mg protein) was recorded in late season and the minimum was detected in early season (0.35 ± 0.08 nmol/mg protein) (Figure 1). The high production of TBARS in both species during late spawning may be associated with decreasing spermatozoon motility, as suggested by our previous results (Shaliutina-Kolesova et al. 2016). A significant correlation between the lipid peroxidation and the reduced motility has also been reported in mammalian (Yeni et al. 2010), including human (Aitken 1999) spermatozoa. This may result in a cascade of episodes including LPO of the spermatozoon plasma membrane that ultimately affects axonal protein phosphorylation and leads to spermatozoon immobilization.

The formation of carbonyl groups, although not a specific indicator, is an index of oxidative modification of proteins. An increase in carbonyl content and protein oxidation may occur as the consequence of the attack by free radicals (DuTeaux et al. 2004). Carbonyl groups may be introduced into proteins by primary reactions in which proteins are oxidized by reactive species generated by the oxidation of other molecules. Our previous work by Shaliutina- Kolesova et al. (2016) demonstrated that total protein level varied from 1.85 ± 0.45 to 2.38 ± 0.21 mg/ml in carp, while in rainbow trout the maximum protein level in SP (2.14 ± 0.28 mg/ml) was recorded in mid-phase and the minimum was

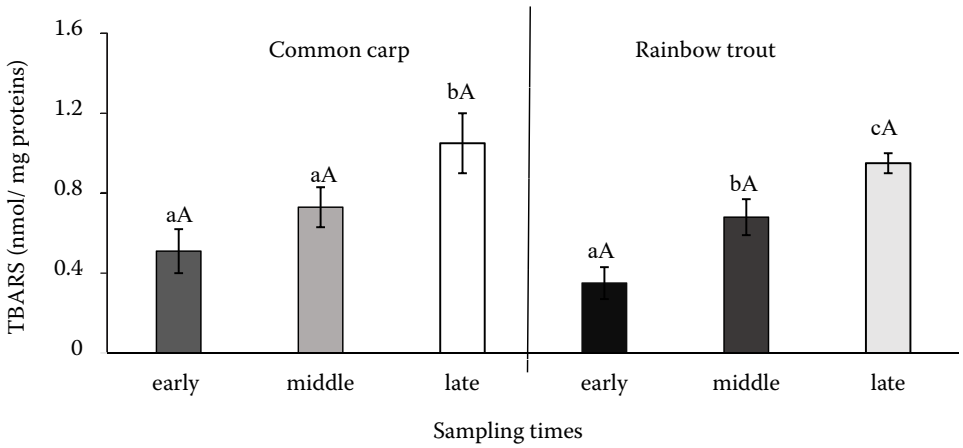

Figure 1. Level of lipid peroxidation (TBARS) in the seminal plasma of common carp *Cyprinus carpio* and rainbow trout *Oncorhynchus mykiss* during the spawning season (carp, $n = 16$ and rainbow trout, $n = 15$). Data are expressed as mean ± SD
[A]species differences between samples collected at the same time point in the spawning season, [a–c]spawning seasonal differences within species; values with the same superscripts are not significantly different (ANOVA, $P > 0.05$)

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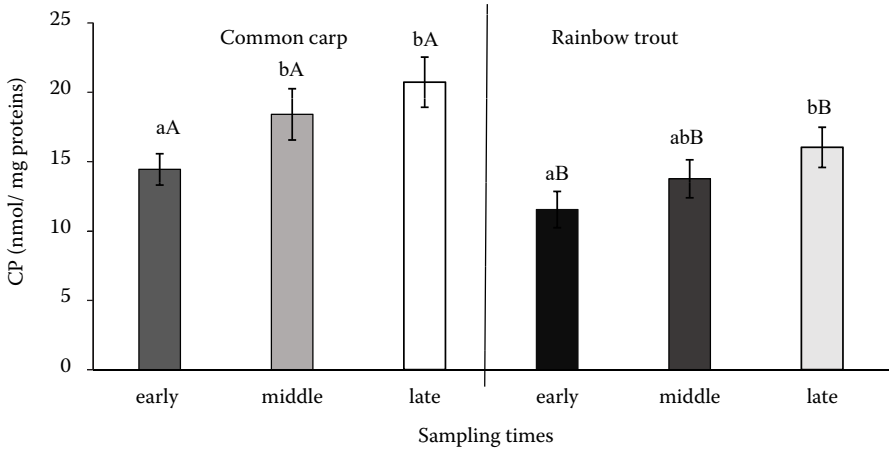

Figure 2. Level of carbonyl protein (CP) in the seminal plasma of common carp *Cyprinus carpio* and rainbow trout *Oncorhynchus mykiss* during the spawning season (carp, $n = 16$ and rainbow trout, $n = 15$). Data are expressed as mean ± SD ^{A,B}species differences between samples collected at the same time point in the spawning season, ^{a,b}spawning seasonal differences within species; values with the same superscripts are not significantly different (ANOVA, $P > 0.05$)

detected in early phase of the reproductive season (1.19 ± 0.25 mg/ml). The present findings showed that the CP content in carp SP significantly increased in mid-spawning, with the highest level of 20.74 ± 1.81 nmol/mg protein in the late phase (Figure 2). The level of CP in rainbow trout sperm also altered with time in the spawning season, with significantly higher CP (16.04 ± 1.45 nmol/mg protein) recorded in late season spawning than in the early phase. In mid phase the CP level was 13.77 ± 1.37 nmol/mg protein and no statistical differences compared to late phase were observed. The rise in CP could be associated with increased testicular activity late in the spawning season. A significantly higher concentration of CP was observed in carp SP samples than in rainbow trout ($P < 0.05$).

Antioxidants act as free radical scavengers to protect spermatozoa against ROS (Agarwal and Said 2005). SOD, a major intracellular enzyme protecting against oxygen toxicity by catalysing the ablation of oxygen, was not shown to be significantly affected by season in either species (Table 1). Nevertheless, the higher concentrations of SOD observed in carp (3.04± 0.35 mU/mg proteins) and rainbow trout (2.45 ± 0.34 mU/mg proteins) SP during late-spawning were apparently related to higher levels of ROS, and not due to a lower antioxidant capacity.

Glutathione plays an important role in intracellular defence against oxidative stress in spermatozoa by reducing hydrogen peroxide to water and lipoperoxides to alkyl alcohols (Gadea et al. 2004). *In vitro* studies have shown that GR preserves tail-beat fre-

Table 1. Antioxidant enzyme activity (mU/mg proteins) in common carp *Cyprinus carpio* ($n = 16$) and rainbow trout *Oncorhynchus mykiss* ($n = 15$) seminal plasma during the reproductive season. Data are expressed as mean ± SD

Species	Parameters (mU/mg protein)	Sampling times during the spawning season		
		early	middle	late
Common carp	SOD	2.47 ± 0.27^{aA}	2.91 ± 0.39^{aA}	3.04 ± 0.35^{aA}
	GR	18.18 ± 1.93^{aA}	20.36 ± 2.02^{aA}	25.17 ± 2.35^{bA}
	GPx	12.36 ± 1.87^{aA}	14.21 ± 2.00^{aA}	16.08 ± 1.96^{aA}
Rainbow trout	SOD	1.62 ± 0.40^{aB}	1.67 ± 0.42^{aB}	2.45 ± 0.34^{aA}
	GR	13.29 ± 2.01^{aB}	24.13 ± 2.8^{bA}	12.34 ± 2.35^{aB}
	GPx	9.18 ± 1.32^{aA}	15.11 ± 1.47^{bA}	18.88 ± 1.55^{bA}

SOD = superoxide dismutase , GR = glutathione reductase , GPx = glutathione peroxidase
^{A,B}differences between samples collected from different fish species at the same time point in the spawning season
^{a,b}differences within samples collected from the same species at different time points
different letters denote significant difference among datasets (ANOVA, $P < 0.05$)

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quency, reduces LPO, and improves spermatozoon membrane characteristics (Shaliutina-Kolesova et al. 2013). Results of this study showed that GR activity in carp SP was the highest in late spawning compared with other sampling times ($P < 0.05$, Table 1). In rainbow trout, GR was significantly higher (24.13 ± 2.8 mU/mg protein) in mid-spawning. We suggest that the increase of GR activity may be responsible for the coincident reduction of toxic products such as lipid hydroperoxides.

GPx catalyzes the reduction of organic and inorganic hydroperoxides by acting as an electron donor. The GPx family consists of several proteins classified according to their sequence, substrate specificity, and subcellular localization (Chabory et al. 2009). Several studies have demonstrated the importance of the GPx family during spermatogenesis and its relationship to male factor fertility (Schneider et al. 2009). In the current study, significantly lower GPx activity (9.18 ± 1.32 mU/mg protein, Table 1) was found in rainbow trout SP in early spawning compared to other sampling times, while no significant differences in GPx were observed in carp over the course of the reproductive period. These findings suggest that GPx could be a useful marker of gonad function in fish during the spawning season. This enzyme might play a pivotal role in the biochemical mechanisms involved in male infertility.

We found season-dependent changes in the antioxidant defence system and the oxidant content of SP of carp and rainbow trout. We suggest that the increased metabolic rates during the late phase of spawning could possibly increase the rate of ROS formation, leading to oxidative stress. The increase of ROS attack results in a decreased spermatozoon motility, in agreement with our previous work (Shaliutina-Kolesova et al. 2016). These results suggest the need for a higher antioxidant protection late in the spawning season, when lower spermatozoon motility and viability were observed.

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