Comparison of oxidant and antioxidant status of seminal plasma and spermatozoa of several fish species

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ABSTRACT: Oxidant and antioxidant activity in seminal plasma was compared with that in spermatozoa of teleost (common carp *Cyprinus carpio* and brook trout *Salvelinus fontinalis*) and chondrostean (Russian sturgeon *Acipenser gueldenstaedtii*, Siberian sturgeon *Acipenser baerii,* and sterlet *Acipenser ruthenus*) fishes. No differences were found between seminal plasma and spermatozoa in the level of thiobarbituric-acid-reactive substance (0.24 ± 0.08 to 0.33 ± 0.04 nmol/mg proteins) in Russian sturgeon, Siberian sturgeon, and sterlet. Carbonyl protein concentration was significantly higher in spermatozoa than in seminal plasma of all studied species. Analyzed antioxidants included superoxide dismutase, glutathione reductase, and glutathione peroxidase activity. Significant differences ($P < 0.05$) were detected between seminal plasma and spermatozoa in total superoxide dismutase (SOD) and glutathione reductase (GR). Total glutathione peroxidase (GPx) activity was significantly higher in brook trout (12.56 ± 3.23 mU/mg proteins) and Russian sturgeon (11.56 ± 3.12 mU/mg proteins) spermatozoa compared to seminal plasma (6.81 ± 1.56 mU/mg proteins in brook trout and 9.56 ± 3.12 mU/mg proteins in Russian sturgeon). This study provides new data on oxidant and antioxidant balance between spermatozoa and seminal plasma that may be of value in the development of methods for artificial reproduction of teleost and chondrostean species.

Keywords: antioxidant enzymes; oxidative stress; fish sperm

Reactive oxygen species (ROS) are generally produced as a by-product of normal aerobic metabolism, involving largely the membrane-linked electron transport processes, redox cascades, and mitochondrial respiration. This production can be aggravated under the influence of unfavourable environmental cues (Bhattacharjee, 2010).

Fish spermatozoa are sensitive to damage by ROS, since they possess limited endogenous antioxidant protection while presenting abundant substrates for free radical attack on unsaturated fatty acids and DNA (Poli et al., 2004; Koppers et al., 2010). When the production of ROS by the sperm mitochondria is excessive, the gamete's endogenous antioxidant defenses are rapidly overwhelmed, and oxidative damage induces lipid peroxidation in the spermatozoa with a resultant loss of fertilizing potential (Aitken et al., 1998; Ong et al., 2002; Agarwal et al., 2003; Baker and Aitken, 2004). In fish spermatozoa as well as in mammalian, ROS are generated endogenously through the process of normal cell respiration, but may also arise from interactions with exogenous sources, such as xenobiotic compounds (Gazo et al., 2013); cryopreservation conditions may also cause an oxidative stress and lead to sub-lethal or lethal spermatozoa damage (Li et al., 2010). Oxidative damage has an important impact on sperm physiology, and the study of its nature and effects is of great importance in the field of

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gamete biology. Unfortunately, most studies on oxidative stress indices in fish focus on a limited number of model species, mainly of the family of the Salmonidae, while on other species only little information is available. Therefore, more detailed information about the oxidative stress indices in spermatozoa and seminal plasma of various fish species are of potential interest for aquaculture applications.

Spermatozoa are protected from oxidative damage by various antioxidants and antioxidant enzymes, which are present in the seminal plasma or in spermatozoa. Low levels of antioxidants cause oxidative stress and may damage or kill sperm cells (Lahnsteiner et al., 2010). In fish sperm, an antioxidant system consists in enzymatic and non-enzymatic components (Liu et al., 1995; Ciereszko et al., 2000; Lahnsteiner et al., 2010) which have significant consequence in maintaining the semen viability. It was shown that substances such as ascorbic acid (Ciereszko and Dabrowski, 1995; Metwally and Fouad, 2009), uric acid (Ciereszko et al., 1999), and α-tocopherol (Martínez-Páramo et al., 2012) are considered as important non-enzymatic components, while superoxide dismutase, glutathione reductase, catalase, and glutathione peroxidase (Li et al., 2010; Martínez-Páramo et al., 2012) constitute enzymatic antioxidant component of fish sperm. Under *in vitro* storage conditions, the efficiency of the semen antioxidant systems is low and not enough effective to protect spermatozoa from reactive oxygen species (Lahnsteiner et al., 2010). Therefore in some previous studies it was demonstrated that addition of molecules with antioxidant capacity to the freezing media provides an effective protection against cold shock and reduces the oxidative damage during sperm preservation (Cabrita et al., 2011). However, the effect of each antioxidant is species-specific and the positive effects found in some species may not be true in others.

The aim of this study was to compare oxidant and antioxidant status of the seminal plasma with that of spermatozoa in several fish species and to assess the capacity of fish seminal plasma to protect spermatozoa from oxidative stress.

Russian sturgeon *(Acipenser gueldenstaedtii)*, Siberian sturgeon (*Acipenser baerii)*, sterlet (*Acipenser ruthenus)*, common carp (*Cyprinus carpio*), and brook trout (*Salvelinus fontinalis*) were selected as model species because of their commercial value.

MATERIAL AND METHODS

Brood stock handling and collection of gametes

Russian sturgeon, Siberian sturgeon, and sterlet were maintained at Fischzucht Rhonforelle GmbH & Co. KG, Gersfeld, Germany. Six male *Acipenser gueldenstaedtii* (7 years old, 6–8 kg), *Acipenser baerii* (6 years old, 5–6 kg), and *Acipenser ruthenus* (6 years old, 2.0–2.5 kg) were used for the study. Before hormone stimulation, fish were kept in hatchery tanks at water temperature of 14–15°C. Spermiation was stimulated by intramuscular injection of carp pituitary powder dissolved in 0.9% (w/v) NaCl solution at 5 mg/kg body weight for Russian and Siberian sturgeon 48 h prior to stripping and 4 mg/kg for sterlet 36 h prior to stripping. Semen was collected from urogenital papilla by aspiration through a plastic catheter (4–7 mm diameter) connected to a 20 ml syringe.

Six mature males of common carp (3 years old, 3.5–4.0 kg) and brook trout (3 years old, 2.0 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). Until experimentation, fish were stocked separately in 4 m^3 tanks at water temperature of 22°C for carp and 7°C for brook trout. Carp were injected with carp pituitary extract at 1 mg/kg, 36 h before sperm collection. No hormone treatment was used for the induction of spermiation in brook trout.

Sperm samples were obtained by abdominal massage and collected directly into 10 ml plastic syringes. Special care was taken to avoid contamination by urine, mucus, faeces, or water. Samples were stored at 0–4°C in closed assay tubes until processing.

Indices of oxidative stress

Sperm samples were centrifuged at 5000 *g* at 4°C for 10 min. The seminal plasma (supernatant) and sperm pellet were suspended in 50mM potassium phosphate (KPi) buffer, pH 7.0, containing 0.5mM EDTA and homogenized in an ice bath using a Sonopuls HD 2070 ultrasonicator (Bandelin Electronic, Berlin, Germany). The homogenate was divided into two portions: one in which thiobarbituric-acid-reactive substances (TBARS) and carbonyl derivatives of proteins (CP) were measured and a second that was centrifuged at 12 000 *g* at 4°C for

30 min to obtain post-mitochondrial supernatant for the antioxidant enzyme activity assay.

The TBARS method as described by Lushchak et al. (2005) was adapted to evaluate lipid peroxidation (LPO) in fish seminal plasma and spermatozoa. The TBARS concentration was calculated by light absorption at 535 nm on a spectrophotometer, using a molar extinction coefficient of 156 mM/cm. The TBARS content was expressed as nmol per mg of protein. Carbonyl derivatives of proteins were detected by reaction with 2,4-dinitrophenylhydrazine (DNPH) according to the method described by Lenz et al. (1989). The amount of CP was measured spectrophotometrically at 370 nm using a molar extinction coefficient of 22 mM/cm, expressed as nmol per mg of protein. All oxidative stress indices were made in triplicate for each sample.

Antioxidant parameters

Total superoxide dismutase (SOD) activity was determined by the method of Marklund and Marklund (1974) involving the autoxidation of pyrogallol and was assessed spectrophotometrically at 420 nm. Glutathione peroxidase (GPx) activity was assayed, based on the rate of NADPH oxidation at 340 nm, by the coupled reaction with glutathione reductase (GR). The specific activity was determined using the extinction coefficient of 6.22 mM/cm (Lawrence and Burk, 1976). GR activity was determined spectrophotometrically, measuring NADPH oxidation at 340 nm (Carlberg and Mannervik, 1975). One unit of SOD activity is defined as the amount of the enzyme needed to effect 50% dismutation of the superoxide radical per min. One unit of GPx or GR activity is defined as the amount of the enzyme that consumes 1 μmol of substrate or generates 1 μmol of product per min. Activity was expressed as international milliunits (mU) per mg of protein. All parameters of antioxidant activity were made in triplicate for each sample.

Statistical analysis

All values were expressed as means ± SD and analyzed by factorial Analysis of Variance (ANOVA). Statistical comparison was made by ANOVA followed by Tukey's HSD test for each analyzed parameter. All analyses were performed at a significance level of 0.05 using STATISTICA (Version 9.0, 2009) software for MS Windows.

RESULTS

Oxidative stress indices

Non-significant differences (ANOVA; $P > 0.05$) in levels of TBARS were observed between seminal plasma and spermatozoa of Russian sturgeon,

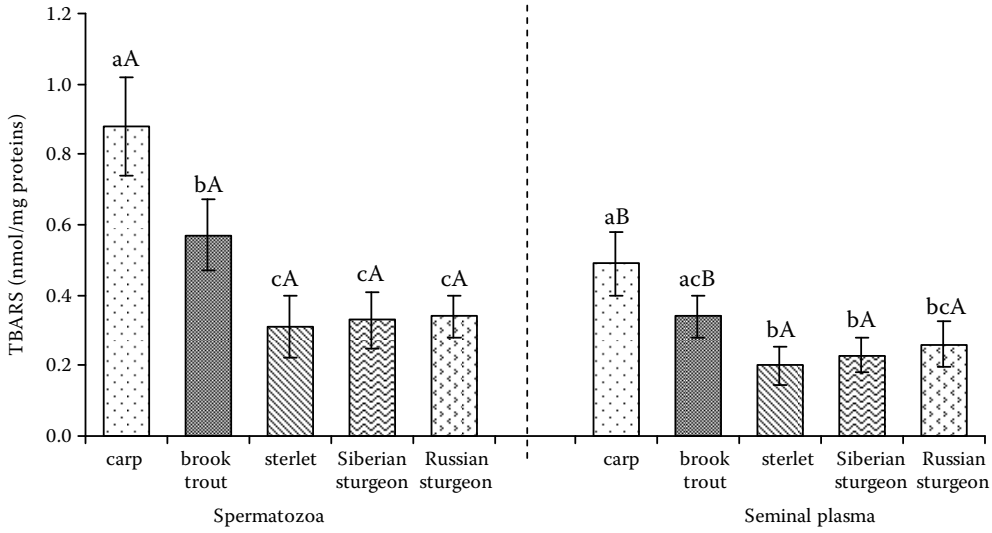

Figure 1. Level of lipid peroxidation (expressed as TBARS) in the seminal plasma and spermatozoa of several fish species (means ± SD)

[A,B]differences between seminal plasma and spermatozoa of a species, [a–c]differences between species; values with the same superscripts are not significantly different ($P > 0.05$, ANOVA)

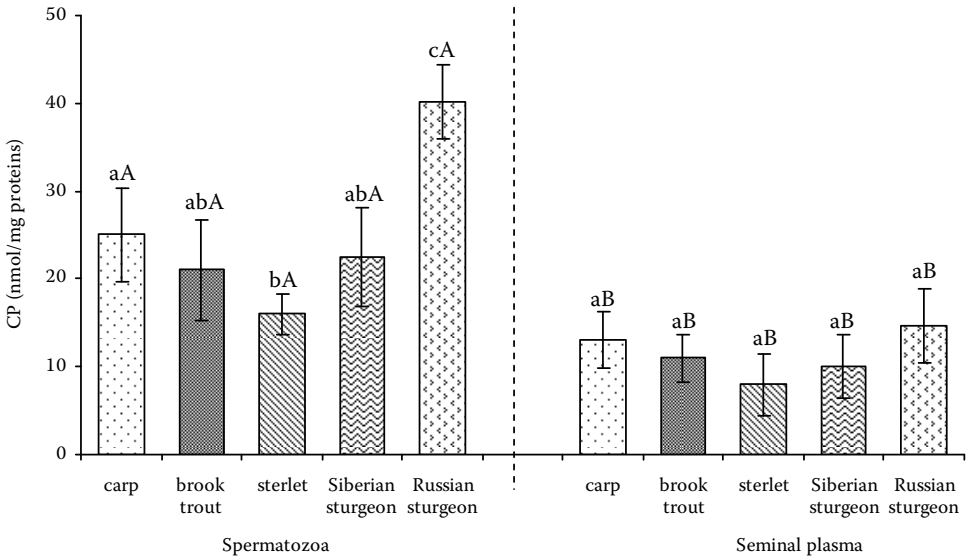

Figure 2. Level of carbonyl protein (CP) in the seminal plasma and spermatozoa of several fish species (means ± SD)
[A,B]differences between seminal plasma and spermatozoa of a species, [a–c]differences between species; values with the same superscripts are not significantly different ($P > 0.05$)

Siberian sturgeon, and sterlet. In carp and brook trout, TBARS levels were significantly higher in spermatozoa (0.88 ± 0.14 and 0.57 ± 0.1 nmol/mg protein, respectively) than in seminal plasma (0.49 ± 0.09 and 0.34 ± 0.06 nmol/mg protein, respectively) (Figure 1). Additionally, in the seminal plasma and spermatozoa of starlet, Russian and Siberian sturgeon the levels of TBARS were lower as compared to carp and brook trout.

Significant differences (ANOVA; $P < 0.05$) in CP concentration were detected between spermatozoa and seminal plasma in all experimental species (Figure 2); however, no significant changes in CP in seminal plasma were found among fish species.

Enzymatic activities

Significant differences (ANOVA; $P < 0.05$) in SOD were detected between spermatozoa and seminal plasma in all studied species. The maximum spermatozoa SOD activity was obtained in Siberian sturgeon (3.6 ± 0.6 mU/mg protein), whereas the minimum was detected in sterlet

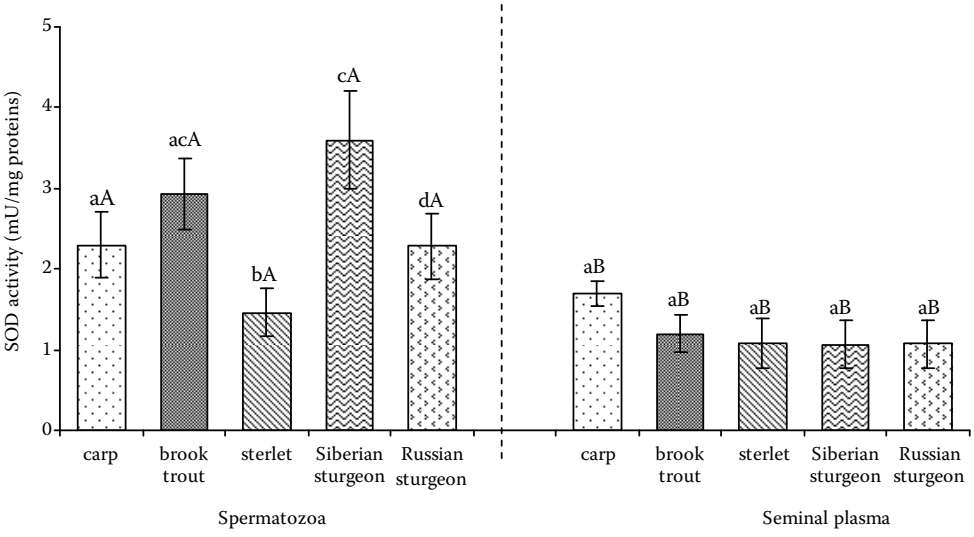

Figure 3. Superoxide dismutase (SOD) activity in seminal plasma and spermatozoa of several fish species (means ± SD)
[A,B]differences between seminal plasma and spermatozoa of a species, [a–d]differences between species; values with the same superscripts are not significantly different ($P > 0.05$)

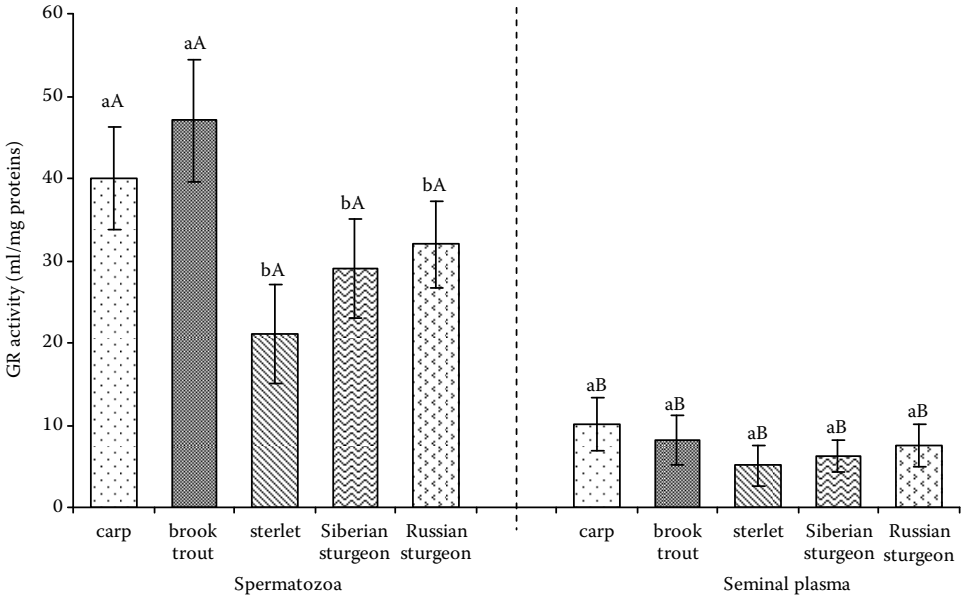

Figure 4. Glutathione reductase (GR) activity in seminal plasma and spermatozoa of several fish species (means ± SD)
[A,B]differences between seminal plasma and spermatozoa of a species, [a,b]differences between species; values with the same superscripts are not significantly different ($P > 0.05$)

(1.08 ± 0.3 mU/mg protein). The mean value of SOD activity in seminal plasma was 1.06–1.7 mU/mg protein. Moreover, there was no significant difference (ANOVA; $P > 0.05$) in SOD of seminal plasma among species (Figure 3).

Glutathione reductase activity showed a trend similar to that of SOD (Figure 4), and statistical differences (ANOVA; $P < 0.05$) in activity of GR between spermatozoa and seminal plasma in all studied species were obtained. The highest value of GR activity was present in the spermatozoa of brook trout (47 ± 7.79 mU/mg proteins) and carp (40 ± 6.33 mU/mg proteins). No significant differences in GR level of seminal plasma were detected among fish species.

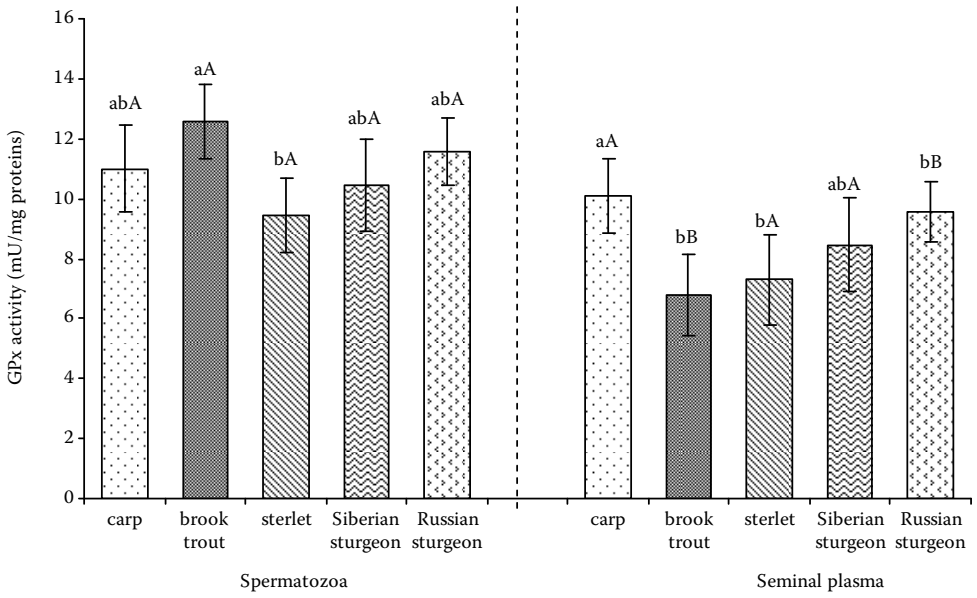

Figure 5. Glutathione peroxidase (GPx) activity in seminal plasma and spermatozoa of several fish species (means ± SD)
[A,B]differences between seminal plasma and spermatozoa of a species, [a,b]differences between species; values with the same superscripts are not significantly different ($P > 0.05$)

High variability in glutathione peroxidase activity in seminal plasma and spermatozoa was observed (Figure 5). Analysis revealed no significant differences (ANOVA; $P > 0.05$) in GPx level between seminal plasma and spermatozoa in carp, sterlet, or Siberian sturgeon. The lowest value of GPx activity (6.81 ± 1.56 mU/mg proteins) was found in brook trout seminal plasma and the highest (12.56 ± 3.23 mU/mg proteins) in brook trout spermatozoa.

DISCUSSION

Antioxidants play an important role in sperm motility, integrity, metabolism, and function, protecting the cells against oxidative damage (Alvarez and Storey, 1983). Their effects have been widely studied in mammalian spermatozoa (Aitken and Baker, 2004); however, a limited amount of information on the precise mechanism of action of antioxidant systems in fish sperm is available. The results of our study demonstrated that in spermatozoa of Russian sturgeon, Siberian sturgeon, sterlet, carp, and brook trout the antioxidant defense levels were higher than in the seminal plasma. Additionally, differences were observed in the levels of oxidative stress response in spermatozoa and seminal plasma. This difference in ROS relative to the antioxidant system of seminal plasma and that of spermatozoa can potentially result in metabolic or functional disorders and a reduction in sperm motility (Li et al., 2009).

The relatively high polyunsaturated fatty acid content of the spermatozoon membrane makes it susceptible to oxidative damage (Trenzado et al., 2006). Lipid peroxidation can lead to a loss of membrane integrity, causing increased cell permeability, enzyme inactivation, resistance to osmotic shock, and decrease in fertilization potential (Shiva et al., 2011). In addition, protein oxidization could have deleterious effects on sperm function, with plasma membrane structure proteins, as well as proteins having enzyme activity, being affected (Domínguez-Rebolledo et al., 2010). Lipid peroxidation is particularly important for aquatic animals, since they normally contain greater amounts of highly unsaturated fatty acids than do other species. Lipid peroxidation has been reported to be a major contributor to the loss of cell function under oxidative stress (Storey, 1996) and has usually been indicated by TBARS level in fish (Oakes and Van der Kraak, 2003). In the current study, oxidative stress, as expressed by TBARS level, was significantly higher in spermatozoa than in seminal plasma, both in carp and brook trout. Additionally, minor differences in the level of TBARS of spermatozoa and that of seminal plasma were found in sterlet and Russian and Siberian sturgeon. However, differences between parameters of antioxidant defense, such as GR activity, were not significant among spermatozoa of these species. This leads to speculation that low TBARS concentration in seminal plasma does not reduce antioxidant activity in spermatozoa. On the other hand, the CP is a result of protein oxidation. The formation of CP is irreversible, causing conformational changes, decreased catalytic activity in enzymes, and ultimately resulting in higher susceptibility of proteins to breakdown by proteases (Zhang et al., 2008). In the present study, we observed that the levels of CP were significantly higher in spermatozoa and varied between all experimental species. In comparison with spermatozoa, there was no significant difference in CP levels of the seminal plasma. This might suggest that variation of CP in spermatozoa is species-specific but can also depend on rearing and environmental conditions. Based on our results, we conclude that the low level of oxidative stress indices in seminal plasma seems to play no role in cellular metabolism (e.g. oxidative phosphorylation) of sperm, which could lead to subsequent decline of motility variables.

In order to cope with the oxidative damage, organisms have evolved multiple systems of antioxidant defense, including enzymatic and non-enzymatic antioxidants (Li et al., 2009, 2010). The major role of antioxidants is implied in the inactivation or transformation of oxidants, which can be either transformed by antioxidant enzymes into less reactive forms or can react with antioxidant molecules that are chemically stable. In the current study, the antioxidant activity was evaluated as total SOD activity, GPx, and GR activity. Superoxide dismutase plays a major role in decreasing LPO and protecting spermatozoa against oxidative damage (Sikka, 1996). Our investigation indicated significantly lower seminal plasma SOD activity in all studied species, as compared to the activity found in spermatozoa. In contrast, several studies reported that SOD is present in high amounts in seminal plasma of all mammalian species, apparently to protect spermatozoa against oxidative stress and possibly also as one of the factors that prevents premature capacitation (de Lamirande et al., 1993; Cassani et al., 2005). Therefore, it can be concluded that

reduced SOD activity in fish seminal plasma might result in reduced protection against ROS.

Glutathione peroxidase, a selenium-containing antioxidant enzyme, removes peroxyl radicals from various peroxides, including H_2O_2, whereas GR regenerates reduced glutathione from its oxidative form (Sikka et al., 2001). We found little difference between the level of GPx activity in spermatozoa and that in seminal plasma in all studied species. Similar results have been reported for *Sparus aurata* (Martínez-Páramo et al., 2009). Li et al. (2010) reported that GPx provided effective protection against cold shock and oxidative damage during cryopreservation of common carp sperm. This is likely to be an adaptive response to toxicant stress and serves to neutralize the impact of increased ROS generation.

The enzyme system comprising GR provides defense against lipid peroxidation in mammalian sperm, and defects in activity of this enzyme can lead to a loss to cell function (Cheema et al., 2009). Our results showed significant differences between seminal plasma and spermatozoa GR level. Several studies have demonstrated that it is possible to reduce damaging effects of ROS by the addition of antioxidant compounds to the freezing media prior to cryopreservation of fish sperm (Lahnsteiner et al., 2011). However, the effect of each antioxidant is species-specific, improving different parameters of sperm quality depending on the type of antioxidant and concentration used (Cabrita et al., 2011).

This study provided new data on the oxidant and antioxidant status of seminal plasma and spermatozoa of Russian sturgeon *(A. gueldenstaedtii)*, Siberian sturgeon (*A. baerii)*, sterlet (*A. ruthenus)*, common carp (*C. carpio*), and brook trout (*S. fontinalis*). The results confirmed that the application of antioxidants to fish seminal plasma could prevent cell injury caused by oxidative stress.

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