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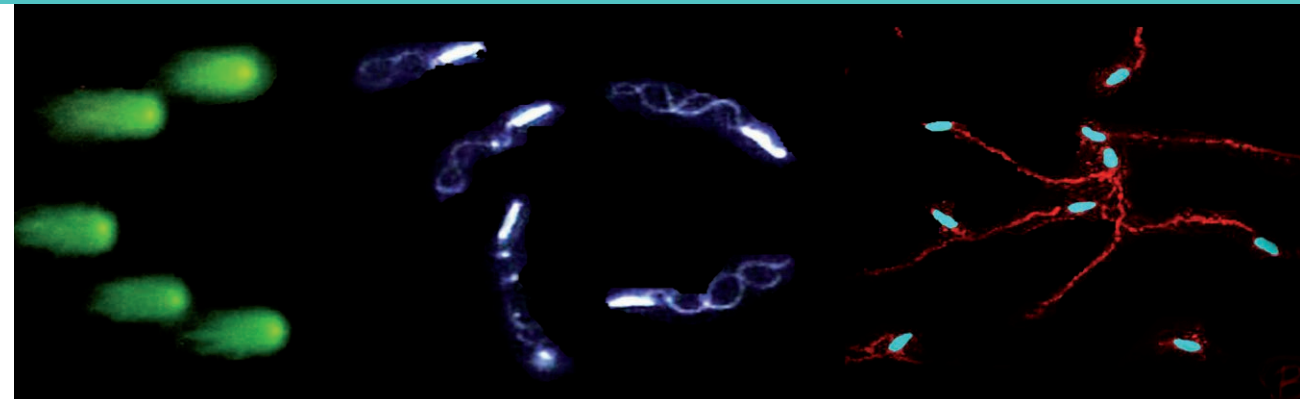a ochrany vod
Faculty of Fisheries
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of Waters

Jihočeská univerzita
v Českých Budějovicích
University of South Bohemia
in České Budějovice

The role of reactive oxygen species and protein phosphorylation in fish spermatozoa

Vliv reaktivních forem kyslíku a proteinové fosforylace na funkci spermií ryb

Ievgeniia Gazo

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CONTENT

CHAPTER 1

GENERAL INTRODUCTION

GENERAL INTRODUCTION

1.1. Fish sperm and importance of studies on sperm physiology

In the course of evolution numerous fish species developed a huge diversity of reproductive strategies and fertilisation modes. Differences in sperm morphology, as well as aspects of motility activation and regulation are a part of this diversity. There are a vast number of factors that lead to triggering sperm motility among fish species with external fertilization (Cosson, 2004). One common feature for spermatozoa of many fish species is that their spermatozoa are immotile in the testis and seminal plasma (Morisawa and Morisawa, 1990). Motility is induced after spawning, when spermatozoa are released into the aqueous external environment. Differences in ionic concentration between this external environment and the seminal plasma is a first requirement for initiation of spermatozoa motility (Cosson, 2010). Sperm of marine fishes, encounters a hyper-osmotic change, meaning a change to a media with higher osmolality, as they are transferred from the seminal plasma into sea water. In fresh water spawning fishes, sperm experiences an opposite, hypo-osmotic change, i.e. exposure to lower osmolality media. And since in the new environment spermatozoa no have the benefit of the protection of seminal plasma, a number of factors affect their movement in the surrounding medium, such as osmotic pressure, pH, temperature, reactive oxygen species (ROS) and pollutants (Alavi and Cosson, 2005, 2006; Zhou et al., 2006). This situation contrasts with species which have internal fertilization, like mammals, in which the female tract offers better protection for spermatozoa against oxidative and osmotic stress.

Sperm motility is a very important prerequisite leading to the contact and fusion between male and female gametes at fertilization. Despite the fact that the initiation of sperm motility and cell signaling associated with this phenomenon have been well studied in mammals (Tash and Means, 1982) and ascidians (Hozumi et al., 2008), in fish, only a limited number of studies have elucidated the mechanisms of motility initiation and intracellular signaling pathways. Moreover, many freshwater fish species are amongvaluable aquaculture species, and understanding sperm cell morphology andthe signaling network for the very rapid activation of flagellar motility is important, not only for elaboration of suitable medium for cryopreservation, but forartificial propagation.

Spermatozoa of teleostean and chondrostean fishes are composed of a head, a mid-piece and a flagellum. The absence of an acrosome is usual in teleostean spermatozoa (Afzelius, 1978). Within teleosts, temporary acrosome-like structures have been reported in brown trout (*Salmo trutta*) (Billard and Takashima, 1983) and several other species (Jamieson, 1991). In sturgeons and paddlefishes (chondrosteans), the sperm head is elongated and has an acrosome at the anterior part (DiLauro et al., 2000; Psenicka et al., 2007). The typical arrangement of nine pairs of peripheral microtubules and two single central tubules compose the axoneme. Initiation of spermatozoa motility occurs as a rapid response to changes in ionic conditions or extracellular ligands, which lead to intracellular signal transduction (Inaba, 2003). The motility mechanism, referred to as the axoneme (a complex assembly of many protein species), is activated as the end of an intracellular signaling pathway. For proper flagellar wave propagation, activation of the axonemes is achieved by phosphorylation of some of the protein subunits associated with molecular motor dyneins. However, mechanisms of intracellular signaling, as well as factors triggering motility, vary significantly among different species.

In Salmonids for example, which is the present working model, a decrease of extracellular concentration of K^+ ions, occurs at transfer from seminal fluid (rich in K^+ ions) into external fresh water (poor in K^+) initiates the spermatozoan motility when accompanied by an increase

of intracellular concentration of Ca^{2+} ions, leading to membrane hyperpolarization. This hyperpolarization activates adenylyl cyclase activity, which itself increases the intracellular cAMP concentration (Morisawa and Okuno, 1982; Billard et al., 1987; Kho et al., 2001). As a result of this burst of cAMP, activation of cAMP-dependent protein kinase A (PKA) occurs (Kho et al., 2001). The PKA is located in the close vicinity of outer arm dyneins (the motor protein devoted to flagellar movement); this leads to the prompt phosphorylation of a dynein light chain, which is a necessary condition for activation of spermatozoa motility in salmonids (Inaba, 2003).

In Cyprinids, it has been shown that simple K^+ transport across the plasma membrane of spermatozoa is not the factor that is responsible for the activation process. Even though Krasznai et al. (2000) found that potassium channels participate in activation of spermatozoa motility and Ca^{2+} influx was suggested to be involved in the initiation of spermatozoa motility in common carp, the motility activation process has been shown to be independent on cAMP (Cosson and Gagnon, 1988; Krasznai et al., 1995; Krasznai et al., 2000). Thus, the molecular mechanism of motility activation and intracellular signaling in spermatozoa of common carp remains to be better elucidated.

In Acipenserids, less information exists about the effect of K^+ on the motility of spermatozoa. It has been shown that K^+ ions play an important role in maintaining spermatozoa quiescence in seminal plasma of several acipenserids (Alavi et al., 2011; Linhart et al., 2003). The potent mobility of spermatozoa is sensitive to very low K^+ concentrations, and Ca^{2+} ions reverse the K^+ inhibitory effect. In this respect, it is theorized that the mechanism of motility regulation in the spermatozoa of this group of fishes would be similar to that described above for salmonids (Cosson, 2010). However, despite the fact that the role of ions in the activation of sperm motility in sturgeons was investigated as well as participation of cAMP at the motility activation step, there are still many questions that must be answered about the mechanisms and function of intercellular signaling, and the interactions between cAMP, calcium, and protein phosphorylation in fish sperm motility.

Another key point in studies of fish spermatozoa is the effect of different aquatic pollutants, on sperm functions. It is not only essential for preserving biodiversity in nature and protecting stocks in aquaculture, but *in vitro* sperm assays may provide a simple and efficient means for evaluating the potential effects of anthropogenic pollutants on aquatic environment.
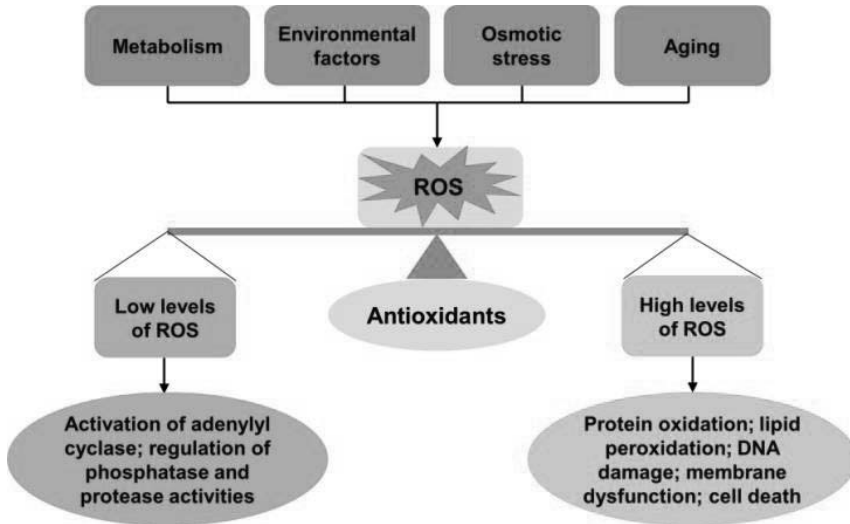
1.2. Sources of reactive oxygen species in sperm

Sperm cells of externally fertilizing aquatic species, including most fish, are particularly vulnerable to oxidative stress when released into the external environment this is because of a minimal protection by their membranes which are in direct contact with the surrounding fluid. Therefore the presence of some pollutants in the environment can lead to ROS production and impair normal reproduction processes of aquatic animals (Zhou et al., 2006).

ROS are short-lived, highly reactive chemical species (atoms or small molecules) that include oxygen ions and peroxides. The high reactivity of ROS is a consequence of unpaired valence-shell electrons, which can generate significant damage to cellular structures. The most common ROS that have potential implications in reproductive biology include superoxide (O^{2-}) anion, hydrogen peroxide (H_2O_2), peroxyl (ROO^-) radical and the very reactive hydroxyl (OH^-) radical. In fish and mammalian spermatozoa, ROS can be generated in two main ways: 1) endogenously, through the process of normal cell respiration; 2) from interactions with exogenous sources (Fig. 1).

Figure 1. *The sources of reactive oxygen species (ROS) and their possible effects on spermatozoa.*

In mammalian spermatozoa, ROS are normally generated endogenously through the process of cell respiration (Aitken et al., 1998a; Ray et al., 2012). The possible main source in most cells is leakage of electrons from the inner mitochondrial membrane during oxidative phosphorylation and ATP generation (Ford, 2004; Ray et al., 2012). ROS can be produced by intracellular oxidase and peroxidase enzymes, such as xanthine oxidase, or as an alternate product by other enzymes such as cytochrome p450 and nitric oxide synthase. Moreover, spermatozoa are capable of generating ROS by a mechanism that involves an NADPH oxidase in the sperm membrane (Aitken et al., 1992). NADPH oxidases are plasma enzymes (Babior, 1999) that catalyse the generation of ROS by allowing electrons to flow from NADPH across the cellular membrane to molecular oxygen, in order to form superoxide (Fulton, 2009) (Fig. 2).

$$NADPH + 2O_2 \leftrightarrow NADP^+ + 2O_2^- + H^+$$
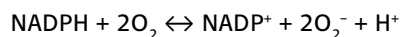
Figure 2. *Transfer of electrons from NADPH through the NADPH oxidase system, creating superoxide, NADP+ and proton (Schirmer et al., 1983).*

Spermatozoa in freshwater are subjected to a significant osmotic challenge when transferred from the seminal fluid to the external environment (Morisawa et al., 1983; Alavi and Cosson, 2006). Hypotonic shock can induce an increase in ROS production and oxidative stress (McCarthy et al., 2010). The exact mechanism of increased ROS generation in response to osmotic stress is unclear; however, work with other cell types suggests that hypo-osmotic cell swelling activates the membrane-associated phospholipase A_2. Activation of phospholipase A_2 may then lead to an activation of the NADPH oxidase complex, resulting in increased production of O_2 (Lambert, 2003). Moreover, cell swelling most probably reflects a shift in protein tyrosine phosphorylation due to an accompanying stimulation of protein tyrosine kinase activity and inhibition of protein tyrosine phosphatase activity by ROS (see § 1.4; Hecht and Zick, 1992; Lambert, 2003).

1.2.1. The effect of water pollution

Complex mixtures of various organic and inorganic environmental pollutants, such as polynuclear aromatic hydrocarbons (PAHs), polychlorobiphenyls (PCBs), quinones, aromatic nitro compounds, aromatic hydroxylamines, bipyridyls and transition metals, are known to elevate production of ROS (Livingstone, 2001). Aquatic habitats throughout the world receive a great amount of pollutants from industrial discharge, agricultural runoff, human and animal waste, municipal and domestic effluents, spillage of vessels and oil spill (Burkholder et al., 2007; Valavanidis et al., 2006). Fish, inhabiting these areas, are chronically exposed to xenobiotics, and can store significant quantities of them in their bodies. Xenobiotic bioaccumulation of such as endocrine disrupting chemicals (EDCs), can affect sex hormone levels and normal gonad development, gamete production and morphology (Table 1). Xenobiotics present in the gonads can impact the quality of sperm during maturation and also when they are stored in the testes.

Foreign substances that pollute aquatic basins can have a direct impact on the ability of the sperm to fertilize eggs. Different xenobiotics have various mechanisms of action, one of which is an increased ROS production (Table 1). It has been proven that xenobiotics can impair fish sperm motility parameters, DNA integrity and induce membrane lipid peroxidation (Zhou et al., 2006).

Because sperm quality is closely related to fertilization success, hatching of embryos and subsequent survival of offspring (Au et al., 2002, 2003), it is particularly important to study the consequences of sperm exposure to foreign compounds in the surrounding medium. Therefore, experiments were conducted in the current work to investigate the effects of short-term *in vitro* exposure to xenobiotics on quality parameters of sturgeon sperm.

1.3. Free radicals: their beneficial and harmful influences on sperm function

Excessive levels of ROS from both exogenous and endogenous sources have been shown to be harmful to spermatozoa. Because ROS are highly reactive, when they reach pathological levels they exert significant damage to biomolecules such as proteins, lipids and nucleic acids. However, besides the pathological effects that are a consequence of excess ROS production, physiological levels are actually essential for proper sperm function (O'Flaherty et al., 2006a). ROS play an important role in transducing signals in the complex biochemical cascades of the spermatozoa. The positive effects of low concentrations of ROS have been documented in mammalian sperm functions, such as capacitation (de Lamirande and Gagnon, 1993; Leclerc et al., 1997), acrosome reaction (Griveau et al., 1995), hyperactivation (de Lamirande and Gagnon, 1993) and sperm–oocyte fusion (Aitken et al., 1995; 1998a). Nevertheless, the specific ROS involved in the activation of these processes is still somewhat controversial and may be species- specific. The effects of different ROS levels on spermatozoa are summarized in Figure 1.

According to present theories, cellular damage results from an improper balance between ROS generation and scavenging activities, which tend to reduce the concentration of these ROS, i.e. a situation in which there is a shift towards pro-oxidants, because of either surplus ROS or diminished antioxidants (Aitken et al., 1998b; Sanocka and Kurpisz, 2004). Normally, within a cell, many defence mechanisms limit the levels of reactive oxidants and, consequently, the damage they inflict (Ames et al., 1993). Enzymes such as superoxide dismutase, catalase, and glutathione peroxidase belong to these defence strategies. Although an antioxidant defense system has been shown to be active in spermatozoa of some fish species, its activity is limited by the amount of cytoplasm, which in a sperm cells is usually very low (Shiva et al.,

2011). Moreover, fish seminal plasma possess a number of enzymatic and non-enzymatic components (Liu et al., 1995; Ciereszko et al., 2000; Lahnsteiner et al., 2010) that play a key role in spermatozoan vitality during their storage in the reproductive system. When ROS levels overwhelm the antioxidant defence system, either through an increase in ROS levels or through a decrease in the cellular antioxidant capacity, oxidative stress occurs.

1.3.1. ROS-induced damage of spermatozoa

Oxidative stress is toxic to spermatozoa as it leads to lipid peroxidation of the plasma membrane, thus, compromising cell functions, including DNA integrity and protein conformation (Agarwal et al., 2003; Agarwal and Said, 2005). Oxidative stress develops in cells due to a tendency of ROS to cause a chain reaction, in such a manner that "radical begets radical". By the reaction of O_2^- and H_2O_2 in the Haber-Weiss reaction (Fig. 3) an $HO^\bullet$ can be produced (Kelly et al., 1998). The production and toxicity of $HO^\bullet$ are of particular biological importance, since $HO^\bullet$ is one of the most potent oxidants, reacting directly with cellular components such as lipids, DNA, and proteins.

$$^\bullet O_2^- + H_2O_2 \rightarrow HO^\bullet + OH^- + O_2$$

Figure 3. *Haber-Weiss reaction.*

The lipid composition of spermatozoan plasma membranes is markedly different from those of somatic cells. They have very high levels of phospholipids, sterols, saturated and polyunsaturated fatty acids, therefore, sperm cells are particularly susceptible to the damage induced by excessive ROS release (Sanocka and Kurpisz, 2004; Griveau et al., 1995). Lipid peroxidation is particularly dangerous for aquatic animals, since they normally contain greater amounts of polyunsaturated fatty acids (PUFAs) than do other species. PUFAs serve as excellent substrates for lipid peroxidation because of the presence of active bis-allylic methylene groups (Kelly et al., 1998). Lipid oxidation has been reported to be a major contributor to the loss of cell function under oxidative stress (Storey, 1996) and trigger the loss of membrane integrity, causing increased cell permeability, enzyme inactivation, structural damages of DNA, and cell death (Aitken et al., 1993; McCarthy et al., 2010; Shiva et al., 2011).

DNA is another key cellular component that is particularly susceptible to oxidative damage. Aitken et al. (1998b) showed that oxidative stress, achieved by incubating human spermatozoa in the presence of NADPH or H_2O_2, was associated with a dramatic decline in the genomic integrity of the cell. Also a variety of other mechanisms of ROS-induced DNA damage have been reported in the literature (reviewed by Cooke et al., 2003). Peroxidation of DNA may result in chromatin cross-linking, base changes, and DNA strand breaks. Moreover, a number of studies have proven that DNA damage of spermatozoa is linked to male infertility (Barroso et al., 2000; O'Brien and Zini, 2005), reduced growth, abnormal development and reduced survival of embryos, larvae and adults of bivalves and urchins (Steinert, 1999; Lesser et al., 2003). In fish spermatozoa, different preservation techniques (short-term storage and cryopreservation), as well as water pollutants have been shown to induce DNA damage (Perez-Cerezales et al., 2009; Zhou et al., 2006). The sensitivity of DNA integrity to low levels of ROS makes it an effective indicator of oxidative stress.

Protein oxidation is particularly detrimental for spermatozoa as the resulting conformational changes to protein structures can render damaged proteins inactive or lead to functional abnormalities. Mechanism of ROS-induced alteration of protein function has been described by Ray et al. (2012). The oxidative interface consists mainly of the redox regulation of redox-

reactive cysteine (Cys) residues on proteins by ROS. Furthermore, redox cycling cations such as Fe^{2+} or Cu^{2+} can bind to cation-binding domains on proteins and transform side-chain amine groups on several amino acids (i.e. lysine, arginine, proline or histidine) into carbonyls. These oxidative modifications result in changes to the structure and/or function of the protein.

It has been showed that excessive ROS production also affects mitochondria functions. In mammals, accumulation of peroxidation products in mitochondria lead to a decrease in ATP production and compromises the maintenance of cell homeostasis (Chance et al., 1979). The inner and outer mitochondrial membrane can be disrupted by a high level of ROS, leading to the activation of caspase, a series of cytosolic cysteine proteases which play a role in apoptosis induction (Agarwal et al., 2003).

Fish spermatozoan movement primarily depends on initial ATP content, plasma membrane potential for triggering the flagellar beating and Ca^{2+} signaling (Cosson, 2010). Successful delivering of paternal DNA into the oocyte is a major function of spermatozoa. Therefore, any modification of these key elements by oxidative stress can significantly affect fertilization success or embryo development. Considering that sperm storage, as well as the number of pollutants, present in open waters, may induce increased ROS level in fish spermatozoa, investigations of the effects of oxidative stress and antioxidants on fish spermatozoa are of great importance for better understanding of mechanisms of cellular damage and possible ways for sperm protection.

1.3.2. Reactive oxygen species in sperm intracellular signaling pathways

As was mentioned above, studies with human and mammalian spermatozoa have demonstrated that low concentrations of ROS have a positive effect on sperm capacitation, acrosome reaction, and sperm–oocyte fusion, suggesting that the signaling pathways underlying these processes may actually be under redox control. A key step in the signaling pathways, controlling sperm capacitation and the acrosome reaction, is believed to be protein tyrosine phosphorylation (Baumber et al., 2003) (principles of protein phosphorylation are described below in § 1.4). A number of studies support the role of ROS in the promotion of tyrosine phosphorylation that have been observed during mammalian sperm capacitation (Aitken et al., 1998a; O'Flaherty et al., 2006a; 2006b). For example, the exogenous addition of ROS (O_2^-, H_2O_2, and NO^-) may stimulate adenylyl cyclase (AC) and cause an immediate generation of cAMP in spermatozoa (O'Flaherty et al., 2006b). Consequently an increase of cAMP concentration leads to activation of protein kinase A, which plays a key role in the mechanisms that trigger capacitation and the associated protein tyrosine phosphorylation in mammalian species (Aitken et al., 1998a; Visconti et al., 1995; Leclerc et al., 1997). Free radicals also induce phosphorylation of such proteins as MEK-like protein and threonine–glutamine–tyrosine motif (O'Flaherty et al., 2006a). Thus, the major phosphorylation pathways known to occur during mammalian sperm capacitation can be modulated by ROS.

One of the mechanisms by which ROS affect transduction elements and pathways is by redox modulation of the sulfhydryl: disulfide pair on proteins (Ray et al., 2012). Protein phosphatases are a good example of transduction factors modulated by this mechanism. Thus, the ROS-mediated regulation of protein tyrosine phosphorylation depends on the balance between activities of protein tyrosine kinase (PTK) and protein tyrosine phosphatase (PTP) (see § 1.4; Barford, 2004). Other targets of ROS are protein Ser/Thr phosphatases, such as PP1 and PP2A inactivated via oxidation of cysteine residues (Hecht and Zick, 1992). Therefore, it can be suggested that ROS are involved in the modulation of PKA-dependent phosphorylation, related to capacitation, by both activating the kinases and inactivating protein phosphatases. ROS can also selectively and reversibly inactivate the ERK protein phosphatases, such as MKP-

3, as it was shown in neuron activation (Muda et al., 1996). However, the redox regulation of those pathways in fish spermatozoa has not yet been demonstrated.

In the marine teleost, gilthead seabream (*Sparus aurata*), a recent study revealed that upon activation of spermatozoa in hyperosmotic conditions, the teleost orthologue of aquaporin-8, termed Aqp8b, is rapidly phosphorylated and inserted into the inner mitochondrial membrane (Chauvigné et al., 2015). Aquaporins are integral membrane proteins from a larger family of major intrinsic proteins that form pores in the membrane of biological cells and facilitate water transport across the membrane. Chauvigné et al. (2015) showed that in spermatozoa of gilthead seabream, Aqp8b mediates H_2O_2 efflux from mitochondria and mitigates cellular oxidative stress. Hence ROS signaling exhibits a complex relationship with protein phosphorylation, where redox-regulation is involved in modification of balance between kinases and phosphatases, and, vice versa, protein phosphorylation can regulate ROS production.

The role of ROS in fish sperm motility is not yet clear. However, it has been shown that ROS are able to modulate swimming behavior in green alga *Chlamydomonas reinhardtii* (Wakabayashi et al., 2011). Furthermore, presence of low doses of ROS stimulated human sperm hyperactivation (de Lamirande and Gagnon, 1993). Previous studies showed that the outer arm dynein of sea urchin sperm axoneme contains thioredoxin, a small redox protein that plays an important role in ROS-response (Ogawa et al., 1996). Thus, it can be suggested that ROS may have a positive effect on spermatozoa motility.

ROS can function as second messengers that modulate spermatozoa functions in mammalian sperm cells (Ray et al., 2012). However, very little is known presently about the role of ROS in intracellular signaling in fish spermatozoa.

1.4. Protein phosphorylation in spermatozoa

The one of the most important processes in sperm intracellular signaling is a protein post-translational modification. Spermatozoa are transcriptionally inactive cells, which means that there is no possibility of protein synthesis after maturation. Protein phosphorylation is a type of post-translational modifications that was the most extensively studied in mammalian spermatozoa (O'Flaherty et al., 2006b). However, in fish spermatozoa, phospho-proteins involved in motility have been identified only for few species, as well as factors involved in intracellular signaling and regulating the phosphorylation / dephosphorylation, such as Ca^{2+} and cAMP (Table 2).

The phosphorylation state of phosphoproteins is reversibly controlled by the activity of protein kinases and phosphatases. Protein kinases catalyse the transfer of the γ-phosphate from ATP to specific amino acids in proteins. Phosphorylation occurs on serine (Ser), threonine (Thr) and tyrosine (Tyr) residues (Cox and Phillips, 2007). For a large subset of proteins, phosphorylation is tightly associated with protein activity and is a key point of protein function regulation. Phosphorylation regulates protein function and cell signaling by causing conformational changes in the phosphorylated protein. These changes can affect proteins in two ways. First, conformational changes regulate the catalytic activity of a protein so that a protein can be either activated or inactivated by phosphorylation. Second, phosphorylated proteins recruit neighboring proteins which have structurally conserved domains that recognize and bind to phosphomotifs. These domains show specificity for distinct amino acids. In general, processes regulated by protein phosphorylation include capacitation, motility and the acrosome reaction, all of which are required for spermatozoa to reach and fuse with the oocyte (O'Flaherty et al., 2006b).

Knowledge about the phosphorylation pattern in spermatozoa and its regulation during

gamete interaction remains one of the most important issues in this area of investigation. Hence, in the present study, special attention was given to changes in spermatozoa phosphorylation pattern during spermatozoa movement and under an oxidative stress.

1.4.1. Axoneme protein phosphorylation and sperm motility

Flagellar movement in spermatozoa is produced by the active sliding between axonemal pairs of doublet microtubules by dyneins. Dyneins are microtubule-dependent force-generating ATPases and play a key role in microtubule sliding (Visconti et al., 1995; Inaba, 2003). Dyneins are observed as a pair of projecting "arms" on doublet microtubules, designated the outer and inner arms. In salmonid sperm, for example, the outer arm dynein is comprised of two heavy chains (~500 kDa), five intermediate chains (60 – 120kDa) and six light chains (8 – 30 kDa) (Inaba, 2003).

Microtubule sliding is modified so as to produce flagellar bending and wave propagation by other axonemal structures, such as the central pair and radial spokes (Witman, 1992). The number of structures in spermatozoa axoneme is known to be regulated by phosphorylation / dephosphorylation. Most of those proteins in fish are still to be characterized, but some phosphoproteins that participate in sperm motility have been described recently (Inaba et al., 1998; Itoh et al., 2003). Thus, it has been shown that dynein is one of the major targets of cAMP-dependent protein phosphorylation (Hamasaki et al., 1991). The most prominent phosphorylations occur in dynein light chains (DLCs). In salmonids, phosphorylation of the DLC as well as the 48-kDa regulatory subunit of PKA appear to be regulated by proteasomes, which are localized near the outer arm dynein (Inaba et al., 1998). These data suggest that PKA is most likely to be anchored near the outer arm dynein, where it is regulated by proteasomes, and that PKA is involved in phosphorylation of the outer arm DLC.

The intracellular localization of PKA is controlled through association with A-kinase-anchoring proteins (AKAPs) (Diviani and Scott, 2001). AKAPs mediate multivalent signal transduction complexes by simultaneously interacting with multiple signaling enzymes, such as kinases or phosphatases. AKAPs can integrate diverse signaling pathways that regulate the phosphorylation of specific cellular substrates (Colledge and Scott, 1999). Accumulating evidence suggests that AKAP-mediated organization of kinases and phosphatases is particularly important for the transduction of signals to the axoneme.

In addition, there is a strong correlation between Ca^{2+} signaling and protein phosphorylation in sperm flagella. Axonemes contain several Ca^{2+} binding proteins, such as a light chain of outer arm dynein, a subunit of dynein docking complex (DC3), calmodulin (CaM), and parvalbumins (Inaba, 2007). Parvalbumin was discovered in carp muscle (Henrotte, 1952) and, recently, in carp spermatozoa (Dietrich et al., 2010). It became the first protein to be recognized to possess the EF-hand motif (Kretsinger and Nockolds, 1973). This motif is the most common calcium-binding motif found in proteins (Lewit-Bentley and Réty, 2000). The EF-hand family of Ca^{2+}-binding proteins includes the well known calmodulin (CaM), troponin C and S100 proteins. CaM is a multifunctional intermediate messenger protein that transduces Ca^{2+} signals by binding calcium ions and then regulating activity of various target proteins, including protein kinases and phosphatases. It has been shown that Ca^{2+}/CaM activate adenylyl cyclase for cAMP synthesis in sea urchin sperm (Bookbinder et al., 1990). Subsequently, increased cAMP levels lead to activation of PKA and dynein phosphorylation. On the other hand, Tash et al. (1988) showed that CaM-dependent protein phosphatase (calcineurin) also plays an important role in Ca^{2+}-dependent regulation of flagellar motility. It has been proposed that in motile flagellar systems, proteins are in a net phosphorylated state and that regulation may involve carefully coordinated dephosphorylation mediated by CaM. However, the role

of calmodulin, Ca^{2+} signaling and its implication in protein phosphorylation pathways in fish spermatozoa still needs to be studied.

Another important enzyme that participates in sperm motility is a protein kinase C (PKC), a serine/threonine kinase. In human spermatozoa, PKC has been shown to localise in patches along the mid-, principal and end pieces of the flagellum, demonstrating a close association of PKC with flagellar axonemes and outer dense fibers (Kalina et al., 1995). In sea urchin spermatozoa it has been reported that PKC has a key role in the signal transduction mechanisms during initiation and maintenance of motility (White et al., 2007). Moreover, an increase in phosphorylation levels of PKC substrates was observed after motility activation, suggesting that PKC activity and phosphorylation of its target proteins are tightly associated with maintenance of motility (White et al., 2007). Nevertheless, there is a lack of knowledge about the nature of the substrate(s) of PKC in sperm and its role in motility.

1.4.2. Protein phosphorylation in metabolic processes and energy production

Successful sperm motility activation and its maintenance depend on energy production and ATP stores. In spermatozoa, mitochondria are localized in the midpiece, where metabolic reactions occur, leading to the appearance of high-energy phosphates that are required for beating of flagella. It has been shown that there is a dynamic balance between mitochondrial synthesis and ATP metabolizing enzymes (ATPases) in immotile spermatozoa of common carp (Perchec et al., 1995). Oxidative phosphorylation (OXPHOS) and cycle of tricarboxylic acid might be the key metabolic pathway in non-activated fish spermatozoa (Lahnsteiner et al., 1993; Mansour et al., 2003). On the other hand, Nascimento et al. (2008) showed that for motile human spermatozoa, glycolytic pathway is a primary source of energy. Unlike OXPHOS that take place only in midpiece, glycolytic enzymes are localized along all flagellum allowing more efficient transport of ATP to dynein ATPases (Krisfalusi et al., 2006). Thus, it can be suggested that both, oxidative phosphorylation and glycolysis, contribute to sperm motility, but at different stages of sperm maturation. Since cellular energy demand depend on the function and activity of the cell, the adjustment of energy production to physiological demand is essential. In transcriptionally inactive cell, such as spermatozoon, this adjustment is mostly controlled by cell signaling through phosphorylation / dephosphorylation. Recently numerous phosphorylated proteins were identified within the mitochondria and on signaling pathways that act on mitochondria (Hopper et al., 2006; Huttemann et al., 2007). Several studies have concluded that there was PKA activity in the mitochondria of fish spermatozoa (Zilli et al., 2012). Moreover, other studies showed that PKA localization and function in mitochondria are AKAP-mediated (Huttemann et al., 2007).

Numerous data suggest that glycolysis is an important pathway of ATP generation in sperm cells. Glycolysis is the enzyme-catalysed metabolic pathway that converts glucose into pyruvate. Lahnsteiner et al. (1993) reported that spermatozoa of rainbow trout (*Oncorhynchus mykiss*) possess the enzymes involved in glycolysis, phospholipid catabolism and triglyceride metabolism. However, glycolytic pathways vary between spermatozoa of different species by, for example, utilizing different substrates. The main regulating enzymes in glycolysis are hexokinase, phosphofructokinase, and pyruvate kinase. Thus, the entire pathway relies on phosphorylation / dephosphorylation for the appropriate energy production. Moreover, studies on mammalian spermatozoa indicate an activation of glycolytic process in response to increased cAMP levels, suggesting the interplay between sperm motility initiation and activation of glycolysis (Frenkerl et al., 1973).

1.4.3. Protein phosphorylation as a regulator of gamete fusion

It is well known from the studies on mammalian spermatozoa that acrosome reactions and gamete fusion are regulated by protein phosphorylation. A key step in the signaling pathways controlling sperm capacitation and the acrosome reaction is believed to be protein tyrosine phosphorylation (Aitken et al., 1995). Moreover, tyrosine phosphorylation is necessary for the acrosome reaction induced by the zona pellucida, as inhibition of tyrosine phosphorylation prevents the acrosome reaction (Urner and Sakkas, 2003).

Despite the fact that the majority of fish species do not possess an acrosome, there is a lack of knowledge on the mechanisms of sperm-egg fusion in these species. Chondrostean spermatozoa differ from those of teleost in having a functional acrosome that participates in fertilization process (Psenicka et al., 2011). The presence of proteolytic enzymes, such as acrosin and trypsin, in the acrosome of sturgeon spermatozoa has been reported (Psenicka et al., 2009; Ciereszko et al., 1994), as well as the presence of acid phosphatase in sturgeon spermatozoa (Sarosiek et al., 2006). Acid phosphatase in mouse spermatozoa was found to be located in the head region and to play role in acrosome reactions (Poirier, 1975). Nevertheless, the role of protein phosphorylation in mediation and regulation of acrosome reactions in fish spermatozoa has not been studied so far.

1.5. Objectives

The primary objective of this thesis was to address the current lack of knowledge on the role of reactive oxygen species and protein phosphorylation in sperm of commercially important fishes. In particular this thesis focuses on the following four objectives:

Objective I: To investigate the effects of xenobiotic-induced oxidative stress on fish spermatozoa after *in vitro* exposure.

Objective II: To evaluate the protective properties of antioxidants and seminal plasma against oxidative stress in fish spermatozoa.

Objective III: To study the interplay between ROS and protein phosphorylation in fish spermatozoa.

Objective IV: To describe the role of phospho-proteins in the initiation and regulation of spermatozoa movement.

Table 1. *The effect of water pollutants on male fertility of aquatic organisms*

Substance	Concentrations in environmental waters	Tested organisms	Effect on sperm	Mechanism of action	References
Atrazine	0.01–14 µg/L	*Xenopus laevis; Salmo salar; Danio rerio*	*In vivo*: reduced spermatogenesis; feminization of males; lower fertility rate; modified level of hormones in testes	Endocrine disrupting chemical; inhibitor of acetylcholinesterase; interact with cAMP signaling pathway.	Hayes et al., 2010; Moore and Waring, 1998; Suzawa and Ingraham, 2008
Bisphenol A	0.0001–5.1 µg/L	*Poecilia reticulate; Salmo trutta f. fario; Perca fluviatilis; Acipenser ruthenus*	*In vivo*: alterations in sperm concentration after exposure. *In vitro*: decreased spermatozoa motility and velocity; DNA damage; lipid peroxidation and protein carbonylation	Xenoestrogen, binds with estrogen receptors; induce oxidative stress	Hatef et al., 2010; Haubruge et al., 2000; Hulak et al., 2013; Lahnsteiner et al., 2005
Carbamazepine	0.1–1.3 µg/L	*Danio rerio; Cyprinus carpio* L.	*In vivo*: impaired sperm morphology after exposure. *In vitro*: decreased spermatozoa motility and velocity; lipid peroxidation and protein carbonylation	Pharmaceutical; inactivate voltage gated sodium channels; lower the turnover rate of arachidonic acid; induce oxidative stress	Galus et al., 2014; Li et al., 2010a
Duroquinone	Up to 480 µg/L	*Cyprinus carpio* L.; *Acipenser ruthenus*	*In vitro*: sperm DNA damage; decreased spermatozoa motility and velocity; lipid peroxidation and protein carbonylation; decreased hatching rate; decreased ATP content	Xenobiotic; induce ROS production, probably through the NADPH-dependent redox cycling	Linhartova et al., 2013; Zhou et al., 2006
Heavy metals (Hg, Pb, Zn, Cd, Cu and Cr)	Hg: 0.001–5.5 µg/L Pb: 0.005–1.3 mg/L Zn: 0.01–5 mg/L Cd: 0.002–0.2 mg/L Cu: 0.01–55.3 mg/L Cr: 0.005–11 mg/L	*Cyprinus carpio* L.; *Acipenser ruthenus; Dicentrarchus labrax; Gymnotus carapo*	*In vivo*: testes damage, changes in concentration of steroid hormones, reduced sperm count and changes in sperm morphology. *In vitro*: reduced sperm motility; lipid oxidation; changes in enzyme activity	Disruption of the endocrine system; HgCl₂ inhibit water channels; heavy metals induce ROS production and oxidative stress	Abascal et al., 2007; Ebrahimi and Taherianfard, 2011; Li et al., 2010b; Sarosiek et al., 2009; Vergilio et al., 2014

Table 1. *Continued.*

Substance	Concentrations in environmental waters	Tested organisms	Effect on sperm	Mechanism of action	References
Nonylphenol	0.171–1000 µg/L	*Bufo raddei;* *Oryzias latipes;* *Xiphophorus maculatus*	*In vivo:* reduction of GSI, changes in tesitis structure, reduced sperm viability and motility. *In vitro:* reduced sperm motility, membrane integrity and fertilization rate, increased ROS level	Endocrine disrupting chemical, acts as an estrogen; induce ROS production	Feng et al., 2011; Kawana et al., 2003; Kinnberg et al., 2000
Sodium dodecyl sulphate (SDS)	Anionic surfactants: 0.1–150 µg/L	*Cyprinus carpio* L.; *Salmo trutta fario*	*In vitro:* reduced sperm motility, DNA fragmentation	Anionic surfactant; membrane solubilization; modification of enzymes structures and activities	Dietrich et al., 2007
Vinclozolin	0.01–52 µg/L	*Poecilia reticulate;* *Carassius auratus;* *Oryzias latipes;* *Acipenser ruthenus*	*In vivo:* induce demasculinization; affected spermatogenesis and levels of steroid hormones; reduced sperm count, sperm motility and velocity. *In vitro:* reduced sperm motility and velocity; DNA damage; lipid oxidation and protein carbonylation; reduced ATP level	Endocrine disrupting chemical, antiandrogen; induce ROS production	Baatrup and Junge, 2001; Bayley et al., 2002; Chapter 2; Hatef et al., 2012; Kiparissis et al., 2003

Table 2. *Intracellular signaling and protein phosphorylation in spermatozoa of different fish species*

Species	Intracellular signaling	Identified spermatozoa phospho-proteins	Protein functions	References
Cyprinus carpio L.	Ca²⁺-dependent, cAMP-independent	Endoplasmin	Stress response	Krasznai et al., 2000; Li et al., 2013
		Transketolase	Catalyze the pentose phosphate pathway	
		Adenosylhomocysteinase	Hydrolase and catalytic activity,	
Clarias gariepinus	cAMP-independent	Phosphofructokinase; Pyruvate kinase	Metabolism, glycolisis	Mansour et al., 2003;
Morone saxatilis	cAMP-independent	None		He et al., 2004
Oncorhynchus mykiss (Salmo gairdneri)	Ca²⁺-dependent, cAMP-dependent	15-kDa protein; Tctex2-related dynein light chain; cAMP-dependent protein kinase (PKA)	Motility initiation and regulation; intracellular signaling	Hayashi et al., 1987; Inaba, 2002; Itoh et al., 2003; Kho et al., 2001; Morisawa and Okuno, 1982
Oreochromis mossambicus	Ca²⁺-dependent, cAMP-independent	Calmodulin (CaM); Ca²⁺/CaM dependent protein kinase IV (CaMKIV);	Ca²⁺ binding; motility initiation and regulation; intracellular signaling	Morita et al., 2003; Morita et al., 2006
Sparus aurata	Ca²⁺-dependent, cAMP-dependent	A-kinase anchor proteins (AKAP); Novel protein similar to phosphatase and actin regulator 3 of *D. rerio*;	Intracellular sinalling, motility initiation and regulation	Chauvigné et al., 2013; Chauvigné et al., 2015; Zilli et al., 2008
		Aquaporins;	Water transport across membrane	
		Acetyl-CoA synthetase;	Metabolism; ATP production	

REFERENCES

Abascal, F.J., Cosson, J., Fauvel, C., 2007. Characterization of sperm motility in sea bass: the effect of heavy metals and physicochemical variables on sperm motility. J. Fish Biol. 70, 509–522.

Afzelius, B.A., 1978. Fine structure of the garfish spermatozoa. J. Ultrastruct. Res. 64, 309–314.

Agarwal, A., Said, T.M., 2005. Oxidative stress, DNA damage and apoptosis in male infertility: a clinical approach. BJU Int. 95, 503–507.

Agarwal, A., Saleh, R.A., Bedaiwy, M.A., 2003. Role of reactive oxygen species in the pathophysiology of human reproduction. Fertil. Steril. 79, 829–843.

Aitken, R.J., Buckingham, D.W., West, K.M., 1992. Reactive oxygen species and human spermatozoa: analysis of the cellular mechanisms involved in luminol- and lucigenin-dependent chemiluminescence. J. Cell. Physiol. 151, 466–477.

Aitken, R.J., Buckingham, D., Harkiss, D., 1993. Use of a xanthine-oxidase free-radical generating-system to investigate the cytotoxic effects of reactive oxygen species on human spermatozoa. J. Reprod. Fertil. 97, 441–450.

Aitken, R.J., Gordon, E., Harkiss, D., Twigg, J.P., Milne, P., Jennings, Z., Irvine, D.S., 1998b. Relative impact of oxidative stress on the functional competence and genomic integrity of human spermatozoa. Biol. Reprod. 59, 1037–1046.

Aitken, R.J., Harkiss, D., Knox, W., Paterson, M., Irvine, D.S., 1998a. A novel signal transduction cascade in capacitating human spermatozoa characterised by a redox-regulated, cAMP-mediated induction of tyrosine phosphorylation. J. Cell Sci. 111, 645–656.

Aitken, R.J., Paterson, M., Fisher, H., Buckingham, D.W., van Duin, M., 1995. Redox regulation of tyrosine phosphorylation in human spermatozoa and its role in the control of human sperm function. J. Cell Sci. 108, 2017–2025.

Alavi, S.M.H., Cosson, J., 2005. Sperm motility in fishes. I. Effects of temperature and pH: a review. Cell Biol. Int. 29, 101–110.

Alavi, S.M.H., Cosson, J., 2006. Sperm motility in fishes. (II) Effects of ions and osmolality: A review. Cell Biol. Int. 30, 1–14.

Alavi, S.M.H., Gela, D., Rodina, M., Linhart O., 2011. Roles of osmolality, calcium — Potassium antagonist and calcium in activation and flagellar beating pattern of sturgeon sperm. Comp Biochem Physiol A 160, 166–174.

Ames, B.N., Shigenaga, M.K., Hagen, T.M., 1993. Oxidants, antioxidants, and the degenerative diseases of aging. Proc. Natl. Acad. Sci. USA 90, 7915–7922.

Au, D.W.T., Chiang, M.W.L., Tang, J.Y.M., Yuen, B.B.H., Wang, Y.L., Wu, R.S.S., 2002. Impairment of sea urchin sperm quality by UV-B radiation: predicting fertilization success from sperm motility. Mar. Pollut. Bull. 44, 583–589.

Au, D.W.T., Yurchenko, O.V., Reunov, A.A., 2003. Sublethal effects of phenol on spermatogenesis in sea urchins (*Anthocidaris crassipina*). Environ. Res. 93, 92–98.

Baatrup, E., Junge, M., 2001. Antiandrogenic pesticides disrupt sexual characteristics in the adult male guppy (*Poecilia reticulata*). Environ. Health Perspect. 109, 1063–1070.

Babior, B.M., 1999. NADPH oxidase: an update. Blood 93, 1464–1476.

Barford D., 2004. The role of cysteine residues as redox-sensitive regulatory switches. Curr. Opin. Struct. Biol. 14, 679–686.

Barroso, G., Morshedi M., Oehninger, S., 2000. Analysis of DNA fragmentation, plasma membrane translocation of phosphatidylserine and oxidative stress in human spermatozoa. Hum. Reprod. 15, 1338–1344.

Baumber, J., Sabeur, K., Vo, A., Ball, B.A., 2003. Reactive oxygen species promote tyrosine phosphorylation and capacitation in equine spermatozoa. Theriogenology 60, 1239–1247.

Bayley, M., Junge, M., Baatrup, 2002. Exposure of juvenile guppies to three antiandrogens causes demasculinization and a reduced sperm count in adult males. Aquat. Toxicol. 56, 227–239.

Billard, R., Cosson, M.-P., Christen, R., 1987. Some recent data on the biology of trout spermatozoa. In: Idler, D. R., Crim, L. W., Walsh, J. M. (Eds.), Proceedings of the 3rd International Symposium on Reproductive Physiology of Fish. St John's: Memorial University Press pp. 187–190.

Billard, R., Takashima, F., 1983. Resorption of spermatozoa in the sperm duct of rainbow trout during the post-spawning period. Bull. Jpn. Soc. Sci. Fish 49, 387–392.

Bookbinder, L.H., Moy, G.W., Vacquier, V.D., 1990. Identification of sea urchin sperm adenylate cyclase. J. Cell Biol. 111, 1859–1866.

Burkholder, J., Libra, B., Weyer, P., Heathcote, S., Kolpin, D., Thorne, P.S., Wichman, M., 2007. Impacts of waste from concentrated animal feeding operations on water quality. Environ. Health Perspect. 115, 308–312.

Chance, B., Sies, H., Boveris, A., 1979. Hydroperoxide metabolism in mammalian organs. Physiol. Rev. 59, 527–605.

Chauvigné, F., Boj, M., Finn, R.N., Cerdà, J., 2015. Mitochondrial aquaporin-8-mediated hydrogen peroxide transport is essential for teleost spermatozoon motility. Sci. Rep. 5, 7789.

Chauvigné, F., Boj, M., Vilella, S., Finn, R.N., Cerdà, J., 2013. Subcellular localization of selectively permeable aquaporins in the male germ line of a marine teleost reveals spatial redistribution in activated spermatozoa. Biol. Reprod. 89, 37.

Ciereszko, A., Dabrowski, K., Lin, F., Doroshov, S.I., 1994. Identification of trypsin-like activity in sturgeon spermatozoa. J. Exp. Zool. 268, 486–491.

Ciereszko, A., Glogowski, J., Dabrowski, K., 2000. Biochemical characteristics of seminal plasma and spermatozoa of freshwater fishes. In: Tiersch, T.R., Mazik, P.M. (Eds), Cryopreservation in aquatic species. World Aquaculture Society, Baton Rouge, pp 20–48.

Colledge, M., Scott, J.D., 1999. AKAPs: from structure to function. Trends Cell Biol. 9, 216–221.

Cooke, M.S., Evans, M.D., Dizdaroglu, M., Lunec, J., 2003. Oxidative DNA damage: Mechanisms, mutation, and disease. FASEB J. 17, 1195–1214.

Cosson, J., 2004. The ionic and osmotic factors controlling motility of fish spermatozoa. Aquaculture International 12, 69–85.

Cosson, J., 2010. Frenetic activation of fish spermatozoa flagella entails short-term motility, portending their precocious decadence. J. Fish Biol. 76, 240–279.

Cosson, M.-P., Gagnon, C., 1988. Protease inhibitors and substrates block motility and microtubule sliding of sea urchin and carp spermatoza. Cell. Motil. Cytoskeleton 10, 518–527.

Cox, M.M., Phillips, G.N., 2007. Handbook of Proteins: Structure, Function and Methods. John Wiley & Sons, Ltd., Chichester, UK.

de Lamirande, E., Gagnon, C., 1993. A positive role for the superoxide anion in triggering hyperactivation and capacitation of human spermatozoa. J. Androl. 16, 21–25.

Dietrich, G.J., Zabowska, M., Wojtczak, M., Słowińska, M., Kucharczyk, D., Ciereszko, A., 2007. Effects of different surfactants on motility and DNA integrity of brown trout (*Salmo trutta fario*) and common carp (*Cyprinus carpio*) spermatozoa. Reprod. Biol. 7, 127–142.

DiLauro, M.N., Kaboord, W.S., Walsh, R.A., 2000. Sperm-cell ultrastructure of North American sturgeon I. The Atlantic sturgeon (*Acipenser oxyrhynchus*). Can. J. Zool. 78, 438–447.

Diviani, D., Scott, J.D., 2001. AKAP signaling complexes at the cytoskeleton. J Cell Sci. 114, 1431–1437.

Ebrahimi, M., Taherianfard, M., 2011. The effects of heavy metals exposure on reproductive systems of cyprinid fish from Kor River. Iran. J. Fish. Sci. 10, 13–24.

Feng, M., Chen, P., Wei, X., Zhang, Y., Zhang, W., Qi, Y., 2011. Effect of 4-nonylphenol on the sperm dynamic parameters, morphology and fertilization rate of *Bufo raddei*. Afr. J. Biotechnol. 10, 2698–2707.

Ford, W.C.L., 2004. Regulation of sperm function by reactive oxygen species. Hum. Reprod. Update 10, 387–399.

Frenkerl, G., Peterson, R.N., Freund, M., 1973. The role of adenine nucleotides and the effect of caffeine and dibutyryl cyclic AMP on the metabolism of guinea pig epididymal spermatozoa. Proc. Soc. Exp. Biol. Med. 144, 420–425.

Fulton, D.J.R., 2009. Nox5 and the regulation of cellular function. Antioxid. Redox Signal. 11, 2443–2452.

Galus, M., Rangarajan, S., Lai, A., Shaya, L., Balshine, S., Wilsona, J.Y., 2014. Effects of chronic, parental pharmaceutical exposure on zebrafish (*Danio rerio*) offspring. Aquat. Toxicol. 151, 124–134.

Griveau, J. F., Dumont, E., Renard, P., Callegari, J.P., Le Lannou D., 1995. Reactive oxygen species, lipid peroxidation and enzymatic defence systems in human spermatozoa. J. Reprod. Fertil. 103, 17–26.

Hamasaki, T., Barkalow, K., Richmond, J., Satir, P., 1991. cAMP-stimulated phosphorylation of an axonemal polypeptide that copurifies with the 22S dynein arm regulates microtubule translocation velocity and swimming speed in *Paramecium*. Proc. Natl. Acad. Sci. USA 88, 7918–7922.

Hatef, A., Alavi, S.M.H., Linhartova, Z., Rodina, M., Policar, T., Linhart, O., 2010. *In vitro* effects of Bisphenol A on sperm motility characteristics in *Perca fluviatilis* L. (Percidae; Teleostei). J. Appl. Ichthyol. 26, 696–701.

Hatef, A., Alavi, S.M.H., Milla, S., Krisťan, J., Golshan, M., Fontaine, P., Linhart, O., 2012. Anti-androgen vinclozolin impairs sperm quality and steroidogenesis in goldfish. Aquat. Toxicol. 122– 123, 181–187.

Haubruge, E., Petit, F., Gage, M.J., 2000. Reduced sperm counts in guppies (*Poecilia reticulata*) following exposure to low levels of tributyltin and bisphenol A. Proc. Biol. Sci. 267 (1459), 2333–2337.

Hayashi, H., Yamamoto, K., Yonekawa, H., Morisawa, M., 1987. Involvement of tyrosine protein kinase in the initiation of flagellar movement in rainbow trout spermatozoa. J. Biol. Chem. 262, 16692–16698.

Hayes, T.B., Khoury, V., Narayan, A., Nazir, M., Park, A., Brown, T., Adame, L., Chan, E., Buchholz, D., Stueve, T., Gallipeau, S., 2010. Atrazine induces complete feminization and chemical castration in male African clawed frogs (*Xenopus laevis*). Proc. Natl. Acad. Sci. USA. 107, 4612–4617.

He, S., Jenkins-Keeran, K., Woods III, L.C., 2004. Activation of sperm motility in striped bass via a cAMP-independent pathway. Theriogenology 61, 1487–1498.

Hecht, D., Zick, Y., 1992. Selective inhibition of protein tyrosine phosphatase activities by H_2O_2 and vanadate *in vitro*. Biochem. Biophys. Res. Commun. 188, 773–779.

Henrotte, J.G., 1952. A crystalline constituent from myogen of carp muscles. Nature 169, 968–969.

Hopper, R.K., Carroll, S., Aponte, A.M., Johnson, D.T., French, S., Shen, R.-F., Witzmann, F.A., Harris, R.A., Balaban, R.S., 2006. Mitochondria matrix phosphoproteome: effect of extra mitochondrial calcium. Biochemistry 45, 2524–2536.

Hozumi, A., Padma, P., Toda, T., Ide, H., Inaba, K., 2008. Molecular characterization of axonemal proteins and signaling molecules responsible for chemoattractant-induced sperm activation in *Ciona intestinalis*. Cell. Motil. Cytoskeleton 65, 249–267.

Hulak, M., Gazo, I., Shaliutina, A., Linhartová, P., 2013. *In vitro* effects of bisphenol A on the quality parameters, oxidative stress, DNA integrity and adenosine triphosphate content in sterlet (*Acipenser ruthenus*) spermatozoa. Comp. Biochem. Physiol. C Toxicol. Pharmacol. 158, 64-71.

Huttemann, M., Lee, I., Samavati, L., Yu, H., Doan, J.W., 2007. Regulation of mitochondrial oxidative phosphorylation through cell signaling. Biochim. Biophys. Acta 1773, 1701–1720.

Inaba, K., 2002. Dephosphorylation of Tctex2-related dynein light chain by type 2A protein phosphatase. Biochem. Biophys. Res. Commun. 297, 800–805.

Inaba, K., 2003. Molecular architecture of the sperm flagella: Molecules for motility and signaling. Zoolog. Sci. 20, 1043–1056.

Inaba, K., 2007. Molecular basis of sperm flagellar axonemes: structural and evolutionary aspects. Ann. N. Y. Acad. Sci.1101, 506–526.

Inaba, K., Morisawa, S., Morisawa M., 1998. Proteasomes regulate the motility of salmonid fish sperm through modulation of cAMP-dependent phosphorylation of an outer arm dynein light chain. J. Cell Sci. 111, 1105–1115.

Itoh, A., Inaba, K., Ohtake, H., Fujinoki, M., Morisawa, M., 2003. Characterization of a cAMP-dependent protein kinase catalytic subunit from rainbow trout spermatozoa. Biochem. Biophys. Res. Commun. 305, 855–861.

Jamieson, B.G.M., 1991. Fish evolution and systematics: evidence from spermatozoa. Cambridge University Press, Cambridge.

Kalina, M., Socher, R., Rotem, R., Naor, Z., 1995. Ultrastructural localization of protein kinase C in human sperm. J. Histochem. Cytochem. 43, 439–445.

Kawana, R., Strüssmann, C.A., Hashimoto, S., 2003. Effect of p-Nonylphenol on sperm motility in Japanese medaka (*Oryzias latipes*). Fish Physiol. Biochem. 28, 213–214.

Kelly, S.A., Havrilla, C.M., Brady, T.C., Abramo, K.H., Levin, E.D., 1998. Oxidative stress in toxicology: established mammalian and emerging piscine model systems. Environ. Health Perspect. 106, 375–384.

Kho, K.H., Tanimoto, S., Inaba, K., Oka, Y., Morisawa M., 2001. Transmembrane cell signaling for the initiation of trout sperm motility: roles of ion channels and membrane hyperpolarization for cyclic AMP synthesis. Zoolog. Sci. 18, 919–928.

Kinnberg, K., Korsgaard, B., Bjerregaard, P., Jespersen Å., 2000. Effects of nonylphenol and 17β-estradiol on vitellogenin synthesis and testis morphology in male platyfish *Xiphophorus maculatus*. J. Exp. Biol. 203, 171–181.

Kiparissis, Y., Metcalfe, T.L., Balch, G.C., Metcalfe, C.D., 2003. Effects of the antiandrogens, vinclozolin and cyproterone acetate on gonadal development in the Japanese medaka (*Oryzias latipes*). Aquat. Toxicol. 63, 391–403.

Krasznai, Z., Márián, T., Balkay, L., Gáspár Jr., R., Trón L., 1995. Potassium channels regulate hypo-osmotic shock-induced motility of common carp (*Cyprinus carpio*) sperm. Aquaculture 129, 123–128.

Krasznai, Z., Marian, T., Izumi, H., Damjanovich, S., Balkay, L., Tron, L., Morisawa, M., 2000. Membrane hyperpolarization removes inactivation of Ca^{2+} channels, leading to Ca^{2+} influx and subsequent initiation of sperm motility in the common carp. Proc. Natl. Acad. Sci. USA 97, 2052–2057.

Kretsinger, R.H., Nockolds, C.E., 1973. Carp muscle calcium-binding protein. II. Structural determination and general description. J. Biol. Chem. 248, 3313–3326.

Krisfalusi, M., Miki, K., Magyar, P.L., O'Brien, D.A., 2006. Multiple glycolytic enzymes are tightly bound to the fibrous sheath of mouse spermatozoa. Biol. Reprod. 75, 270–278.

Lahnsteiner, F., Berger, B., Kletzl, M., Weismann, T., 2005. Effect of bisphenol A on maturation and quality of semen and eggs in the brown trout, *Salmo trutta f. fario*. Aquat. Toxicol. 75, 213–224.

Lahnsteiner, F., Mansour, N., Plaetzer, K., 2010. Antioxidant systems of brown trout (*Salmo trutta f. fario*) semen. Anim. Reprod. Sci. 119, 314–321.

Lahnsteiner, F., Patzner, R.A., Weismann, T., 1993. Energy resources of spermatozoa of the rainbow trout *Oncorhynchus mykiss* (Pisces, Teleostei). Reprod. Nutr. Dev. 33, 349–360.

Lambert, I.H., 2003. Reactive oxygen species regulate swelling-induced taurine efflux in NIH3T3 mouse fibroblasts. J. Membrane Biol. 192, 19–32.

Leclerc, P., de Lamirande, E., Gagnon, C., 1997. Regulation of protein tyrosine phosphorylation and human sperm capacitation by reactive oxygen derivatives. Free Radic. Biol. Med. 22, 643–656.

Lesser, M.P., Kruse, V.A., Barry, T.M., 2003. Exposure to ultraviolet radiation causes apoptosis in developing sea urchin embryos. J. Exp. Biol. 206, 4097–4103.

Lewit-Bentley, A., Réty, S., 2000. EF-hand calcium-binding protein. Curr. Opin. Struct. Biol. 10, 637–643.

Li, P., Hulak, M., Li, Z.H., Sulc, M., Psenicka, M., Rodina, M., Gela, D., Linhart, O., 2013. Cryopreservation of common carp (*Cyprinus carpio* L.) sperm induces protein phosphorylation in tyrosine and threonine residues. Theriogenology 80, 84–89.

Li, Z.-H., Li, P., Rodina, M., Randak, T., 2010a. Effect of human pharmaceutical Carbamazepine on the quality parameters and oxidative stress in common carp (*Cyprinus carpio* L.) spermatozoa. Chemosphere 80, 530–534.

Li, Z.-H., Li, P., Dzyuba, B., Randak, T., 2010b. Influence of environmental related concentrations of heavy metals on motility parameters and antioxidant responses in sturgeon sperm. Chem. Biol. Interact. 188, 473–477.

Linhart, O., Cosson, J., Mims, S.D., Rodina, M., Gela, D., Shelton, W.L., 2003. Effects of ions on the motility of fresh and demembranated sperm of common carp (*Cyprinus carpio*) and paddlefish (*Polyodon spathula*). Fish Physiol Biochem 28, 203–205.

Linhartova, P., Gazo, I., Shaliutina, A., Hulak, M., 2013. The *in vitro* effect of duroquinone on functional competence, genomic integrity, and oxidative stress indices of sterlet (*Acipenser ruthenus*) spermatozoa. Toxicol. In Vitro 27, 1612–1619.

Livingstone, D.R., 2001. Contaminant-stimulated reactive oxygen species production and oxidative damage in aquatic organisms. Mar. Pollut. Bull. 42, 656–666.

Liu, L., Dabrowski, K., Ciereszko, A., 1995. Protective effect of seminal plasma proteins on the degradation of ascorbic acid. Mol. Cell Biochem. 148, 59–66

Mansour, N., Lahnsteiner, F., Berger, B., 2003. Metabolism of intratesticular spermatozoa of a tropical teleost fish (*Clarias gariepinus*). Comp. Biochem. Physiol. B 135, 285–296.

McCarthy, M.J., Baumber, J., Kass, P.H., Meyers, S.A., 2010. Osmotic stress induces oxidative cell damage to rhesus macaque spermatozoa. Biol. Reprod. 82, 644–651.

Moore, A., Waring, C.P., 1998. Mechanistic effects of a triazine pesticide on reproductive endocrine function in mature male atlantic salmon (*Salmo Salar* L.) parr. Pestic. Biochem. Physiol. 62, 41–50.

Morisawa, M., Morisawa, S., 1990. Aquisition and initiation of sperm motility. In: Gagnon, C. (Ed.), Controls of Sperm Motility: Biological and Clinical Aspects. Boca Raton, FL: CRC Press pp. 137–152.

Morisawa, M., Okuno, M., 1982. Cyclic AMP induces maturation of trout sperm axoneme to initiate motility. Nature 295, 703–704.

Morisawa, M., Suzuki, K., Shimizu, H., Morisawa, S., Yasuda, K., 1983. Effect of osmolality and potassium on motility of spermatozoa from freshwater cyprinid fishes. J. Exp. Zool. 107, 95–103.

Morita, M., Takemura, A., Nakajima, A., Okuno, M., 2006. Microtubule sliding movement in tilapia sperm flagella axoneme is regulated by Ca^{2+}/calmodulin-dependent protein phosphorylation. Cell Motil. Cytoskeleton 63, 459–470.

Morita, M., Takemura, A., Okuno, M., 2003. Requirement of Ca^{2+} on activation of sperm motility in euryhaline tilapia *Oreochromis mossambicus.* J. Exp. Biol. 206, 913–921.

Muda, M., Boschert, U., Dickinson, R., Martinou, J.C., Martinou, I., Camps, M., Schlegel, W., Arkinstall, S., 1996. MKP-3, a novel cytosolic protein-tyrosine phosphatase that exemplifies a new class of mitogen-activated protein kinase phosphatase. J. Biol. Chem. 271, 4319–4326.

Nascimento, J.M., Shi, L.Z., Tam, J., Chandsawangbhuwana, C., Durrant, B., Botvinick, E.L., Berns, M.W., 2008. Comparison of glycolysis and oxidative phosphorylation as energy sources for mammalian sperm motility, using the combination of fluorescence imaging, laser tweezers, and real-time automated tracking and trapping. J. Cell. Physiol. 217, 745–751.

O'Brien, J., Zini, A., 2005. Sperm DNA integrity and male infertility. Urology 65, 16–22.

O'Flaherty, C., de Lamirande, E., Gagnon, C., 2006a. Reactive oxygen species modulate independent protein phosphorylation pathways during human sperm capacitation. Free Radic. Biol. Med. 40, 1045 – 1055.

O'Flaherty, C., de Lamirande, E., Gagnon, C., 2006b. Positive role of reactive oxygen species in mammalian sperm capacitation: Triggering and modulation of phosphorylation events. Free Radic. Biol. Med. 41, 528–540.

Ogawa, K., Takai, H., Ogiwara, A., Yokota, E., Shimizu, T., Inaba, K., Mohri, H., 1996. Is outer arm dynein intermediate chain 1 multifunctional? Mol. Biol. Cell. 7, 1895–1907.

Perchec, G., Jeulin, C., Cosson, J., André, F., Billard, R., 1995. Relationship between sperm ATP content and motility of carp spermatozoa. J. Cell Sci. 108, 747–753.

Perez-Cerezales, S., Martinez-Paramo, S., Cabrita, E., Martinez-Pastor, F., de Paza, P., Herraez, M.P., 2009. Evaluation of oxidative DNA damage promoted by storage in sperm from sex-reversed rainbow trout. Theriogenology 71, 605–613.

Poirier, G.R., 1975. Fine structure distribution of non-specific acid phosphatase in the head region of mouse spermatozoa from various regions of the male reproductive tract. J. Reprod. Fertil. 43, 495–499.

Psenicka, M., Alavi, S. M. H., Rodina, M., Gela, D., Nebesarova, J., Linhart, O., 2007. Morphology and ultrastructure of Siberian sturgeon *Acipenser baerii* spermatozoa using scanning and transmission electron microscopy. Biol. Cell 99, 103–115.

Psenicka, M., Kaspar, V., Alavi, S.M.H., Rodina, M., Gela, D., Li, P., Borishpolets, S., Cosson, J., Linhart, O., Ciereszko A., 2011. Potential role of the acrosome of sturgeon spermatozoa in the fertilization process. J. Appl. Ichthyol. 27, 678–682.

Psenicka, M., Vancova, M., Koubek, P., Tesitel, J., Linhart, O., 2009. Fine structure and morphology of sterlet (*Acipenser ruthenus* L. 1758) spermatozoa and acrosin localization. Anim. Reprod. Sci. 111, 3–16.

Ray, P.D., Huang, B.W., Tsuji Y., 2012. Reactive oxygen species (ROS) homeostasis and redox regulation in cellular signaling. Cell Signal. 24, 981–990.

Sanocka, D., Kurpisz, M., 2004. Reactive oxygen species and sperm cells. Reprod. Biol. Endocrinol. 2, 12.

Sarosiek, B., Pietrusewicz, M., Radziwoniuk, J., Glogowski, J., 2009. The effect of copper, zinc, mercury and cadmium on some sperm enzyme activities in the common carp (*Cyprinus carpio* L.). Reprod. Biol. 9, 295–301.

Sarosiek, B., Wysocka, J., Wysocki, P., Kowalski, R., Glogowski, J., 2006. Characteristics of acid phosphatase from Russian sturgeon (*Acipenser gueldenstaedtii*) spermatozoa. J. Appl. Ichthyol. 22, 375–379.

Schirmer, R.H., Schulz, G.E., Untucht-Grau, R., 1983. On the geometry of leukocyte NADPH-oxidase, a membrane flavoenzyme: Inferences from the structure of glutathione reductase. FEBS let. 154, 1–4.

Shiva, M., Gautam, A.K., Verma, Y., Shivgotra, V., Doshi, H., Kumar, S., 2011. Association between sperm quality, oxidative stress, and seminal antioxidant activity. Clin. Biochem. 44, 319–324.

Steinert, S.A., 1999. DNA damage as a bivalve biomarker. Biomarkers 4 (6), 492–496.

Storey, B.T., 2008. Mammalian sperm metabolism: oxygen and sugar, friend and foe. Int. J. Dev. Biol. 52, 427–437.

Storey, K.B., 1996. Oxidative stress: animal adaptations in nature. Braz. J. Med. Biol. Res. 29, 1715–1733.

Suzawa, M., Ingraham, H.A., 2008. The herbicide atrazine activates endocrine gene networks via non-steroidal NR5A nuclear receptors in fish and mammalian cells. PLoS ONE 3, e2117.

Tash, J. S., Means, A. R., 1982. Regulation of protein phosphorylation and motility of sperm by cyclic adenosine monophosphate and calcium. Biol. Reprod. 26, 745–763.

Tash, J.S., Krinks, M., Patel, J., Means, R.L., Klee, C.B., Means, A.R., 1988. Identification, characterization, and functional correlation of calmodulin-dependent protein phosphatase in sperm. J. Cell Biol. 106, 1625–1633.

Urner, F., Sakkas, D., 2003. Protein phosphorylation in mammalian spermatozoa. Reproduction 125, 17–26.

Valavanidis, A., Vlahogianni, T., Dassenakis, M., Scoullos, M., 2006. Molecular biomarkers of oxidative stress in aquatic organisms in relation to toxic environmental pollutants. Ecotoxicol. Environ. Saf. 64, 178–189.

Vergilio, C.S., Moreira, R.V., Carvalho, C.E.V., Melo, E.J., 2014. Effects of *in vitro* exposure to mercury on male gonads and sperm structure of the tropical fish tuvira *Gymnotus carapo* (L.). J. Fish Dis. 37, 543–551.

Visconti, P.E., Moore, G.D., Bailey, J.L., Leclerc, P., Connors, S.A., Pan, D., Olds-Clarke, P., Kopf, G.S., 1995. Capacitation of mouse spermatozoa. II. Protein tyrosine phosphorylation and capacitation are regulated by a cAMP-dependent pathway. Development 121, 1139–1150.

Wakabayashi, K., Misawa, Y., Mochiji, S., Kamiya, R., 2011. Reduction-oxidation poise regulates the sign of phototaxis in *Chlamydomonas reinhardtii*. Proc. Natl. Acad. Sci. U S A. 108, 11280–11284.

White, D., de Lamirande, E., Gagnon, C., 2007. Protein kinase C is an important signaling mediator associated with motility of intact sea urchin spermatozoa. J. Exp. Biol. 210, 4053–4064.

Witman, G.B., 1992. Axonemal dyneins. Curr. Opin. Cell Biol. 4, 74–79.

Zhou, B.S., Liu, W.H., Siu, W.H.L., O'Toole, D., Lam, P.K.S., Wu, R.S.S., 2006. Exposure of spermatozoa to duroquinone may impair reproduction of the common carp (*Cyprinus carpio*) through oxidative stress. Aquat. Toxicol. 77, 136–142.

Zilli, L., Schiavone, R., Storelli, C., Vilella, S., 2008. Molecular mechanisms determining sperm motility initiation in two sparids (*Sparus aurata* and *Lithognathus mormyrus*). Biol. Reprod. 79, 356–366.

Zilli, L., Schiavone, R., Storelli, C., Vilella, S., 2012. Molecular mechanism regulating axoneme activation in marine fish: a review. Intern. Aquat. Res. 4, 2.

CHAPTER 2

INFLUENCE OF ENVIRONMENTALLY RELEVANT CONCENTRATIONS OF VINCLOZOLIN ON QUALITY, DNA INTEGRITY, AND ANTIOXIDANT RESPONSES OF STERLET *ACIPENSER RUTHENUS* SPERMATOZOA

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Influence of environmentally relevant concentrations of vinclozolin on quality, DNA integrity, and antioxidant responses of sterlet *Acipenser ruthenus* spermatozoa

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Chemico-Biological Interactions

Influence of environmentally relevant concentrations of vinclozolin on quality, DNA integrity, and antioxidant responses of sterlet *Acipenser ruthenus* spermatozoa

CrossMark

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ABSTRACT

The effects of vinclozolin (VIN), an anti-androgenic fungicide, on quality, oxidative stress, DNA integrity, and ATP level of sterlet (*Acipenser ruthenus*) spermatozoa were investigated *in vitro*. Fish spermatozoa were incubated with different concentrations of vinclozolin (0.5, 2, 10, 15, 20 and 50 µg/l) for 2 h. A dose-dependent reduction in spermatozoa motility and velocity was observed at concentrations of 2–50 µg/l. A dramatic increase in DNA fragmentation was recorded at concentrations 10 µg/l and above. After 2 h exposure at higher test concentrations (10–50 µg/l), oxidative stress was apparent, as reflected by significantly higher levels of protein and lipid oxidation and significantly greater superoxide dismutase activity. Intracellular ATP content of spermatozoa decreased with increasing concentrations of VIN. The results demonstrated that VIN can induce reactive oxygen species stress in fish spermatozoa, which could impair the sperm quality, DNA integrity, ATP content, and the antioxidant defense system.

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1. Introduction

As the majority of pollutants are discharged into coastal waters [1], fishes inhabiting these areas are often chronically exposed to excessive concentrations of toxic compounds. Apart from possible effects on the endocrine system leading to disruptions in reproductive physiology [2,3], gametes of teleosts with external fertilization can be impacted by the ambient environment. Disruption in gamete development or physiology may have a deleterious effect on reproduction, a prerequisite for survival of any aquatic species. During the past decade, toxicity tests involving fish gametes have received wide attention [4,5]. Several studies have shown that ova are not ideal pollution bio-indicators since, at present, their quality can only be measured through time-consuming fertilization experiments. Moreover, the results of these experiments are subject to variability in sperm and uncontrolled factors in ova, such as over-ripening [6]. Toxicity screening using fish sperm has been considered a simple and valid model, since it does not require expensive sterile cell culture conditions [4].

Sturgeon have been classified as endangered by many international organizations. The population of sturgeon species has drastically declined, primarily due to over-fishing, habitat

destruction, and water pollution [7]. In large species such as sturgeon, it is logistically difficult and costly to conduct toxicity evaluations on broodstock-size animals, and the need for sensitive, rapid toxicity testing has led to the increasing use of sturgeon sperm [5]. Fish spermatozoa are generally characterized by short-term motility, most less than 60 s. Considering the limited time available for fertilization after release of sperm into water, any negative effect on sperm quality may dramatically decrease fertilization success [8–10]. Spermatozoa are highly susceptible to oxidative injury, due not only to a lack of protective cytoplasmic enzymes, but also to the high content of poly-unsaturated fatty acids (PUFA) in their plasma membranes [11]. This leads to a decrease in the potential for successful fertilization and an increase in the probability of intracellular structure damage upon exposure to oxidizing agents [12].

In the present study, we investigated potential adverse effects of the well-characterized anti-androgenic compound vinclozolin (VIN) on sturgeon spermatozoa. Vinclozolin [3-(3,5-dichlorophenyl)-5-methyl-5-vinyl-oxazolidine 2,4-dione] is a fungicide widely used in the United States and Europe to control disease in fruits and vegetables [13]. According to Steeger and Garber [14] the peak estimated environmental concentration of vinclozolin is 52.0 µg/l. It degrades via hydrolysis quickly in neutral water (half-life = 1.3 d). In buffered aqueous solutions half-life of vinclozolin was shown to be temperature and pH dependent, so that in phosphate buffer with pH 7 at 13 °C measured half-life

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was 140 h [15]. In aerobic and anaerobic environments, vinclozolin breaks down via microbial degradation, with half-lives ranging 17.6–134 d [14]. It is rapidly metabolized into two active metabolites, M1 and M2, which have high affinity for the androgen receptor and can block gene expression, causing anti-androgenic effects [16]. Previous studies have shown that VIN inhibits the development and maintenance of a variety of male traits in the guppy (*Poecilia reticulata*), including male coloration patterns, sperm count, and sexual behavior [17,18].

This study was undertaken to investigate effects of short-term (2 h) *in vitro* exposure to VIN on quality parameters and oxidative stress in spermatozoa of the endangered sterlet *Acipenser ruthenus* by analyzing oxidative stress indices, such as lipid oxidation and protein carbonylation (CP) and superoxide dismutase (SOD) activity along with their motility and velocity, ATP content, and DNA integrity.

2. Materials and methods

2.1. Broodstock handling and collection of gametes

Sterlet sperm was obtained from six males (6–7 years old; body weight: 0.5–2 kg; body length: 55 ± 9.3 cm) reared in the aquaculture facility of the Research Institute of Fish Culture and Hydrobiology at the University of South Bohemia, Vodnany, Czech Republic. Prior to hormone stimulation, fish were kept in tanks with water temperature varying between 14 and 15 °C. Spermiation was stimulated by intramuscular injection of carp pituitary powder dissolved in 0.9% (w/v) NaCl at a dose of 5 mg/kg of body weight, 48 h before sperm collection. Semen was collected from the urogenital papilla by aspiration through a plastic catheter (5–7 mm diameter) connected to a 20 ml syringe. Special care was taken to avoid contamination with mucus, feces, or water. Samples were stored on ice (0–4 °C) until processing. Spermatozoa concentration of each of the six males was estimated microscopically (Olympus BX 41) at 200× using a Burker cell hemocytometer.

2.2. Sample preparation

Vinclozolin (3-(3,5-dichlorophenyl)-5-ethenyl-5-methyl-2,4 oxazolidinedione; empirical formula: $C_{12}H_9Cl_2NO_3$; MW: 286.1) (PESTANAL®, analytical standard; Sigma–Aldrich, USA) was dissolved in 96% ethanol and diluted with distilled water to obtain a stock solution of 0.1 g/l. Stock solutions were prepared daily. Fish sperm from six individual males was centrifuged at 300×g, 4 °C for 30 min to remove seminal plasma and diluted with an immobilization medium (20 mM Tris, 30 mM NaCl, 2 mM KCl, pH 8.5) to obtain spermatozoa densities of 5×10^8 cells/ml. The sperm subsamples were then exposed for 2 h to final concentrations of 0.5, 2, 10, 15, 20, and 50 µg/l of VIN at 4 °C. A group exposed to immobilization medium only was used as a control. Each experimental condition was duplicated.

2.3. Spermatozoa motility and velocity recording

Spermatozoa velocity (µm/s, measuring only motile spermatozoa) and percent motile spermatozoa (%) were determined after triggering motility under dark-field microscopy (Olympus BX 50, Japan) (20× objective magnification). For triggering, sperm was diluted 1:5000 in activation medium (10 mM Tris, 10 mM NaCl, 1 mM CaCl₂, pH 8.5). To avoid spermatozoa sticking to the microscope slide, 0.2% (w/v) Pluronic (Sigma–Aldrich, USA) was added to the swimming solution. Spermatozoa motility was recorded with a CCD video camera (SONY DXC-970MD, Japan) mounted on the microscope. Analysis of spermatozoa motility was made in triplicate for each sample.

2.4. Spermatozoa motility and velocity evaluation

Velocity and motility were assessed 10, 30, 60, 90, and 120 s post-activation. The successive positions of the video recorded spermatozoa heads were analyzed from five video frames using Olympus MicroImage software (Version 4.0.1. for Windows with a special macro by Olympus C & S). Velocity and percent motility were calculated from spermatozoa head positions on five successive frames with three colors (frame 1 red, frames 2–4 green, and frame 5 blue). Twenty to 40 spermatozoa were counted for each frame. Those that moved were visible in three colors, while non-moving spermatozoa were white. The percent motile was calculated from the number of white and red cells. Spermatozoa velocity was calculated as µm/s based on length traces of spermatozoa from blue to green and red heads, calibrated for magnification.

2.5. Assessment of DNA damage

DNA integrity was assessed using the Comet assay, or single cell gel electrophoresis assay, following the method described by Li et al. [19]. Unless otherwise stated, molecular grade, DNAse-free reagents (Sigma–Aldrich, USA) were used throughout. Microscope slides (OxiSelect[ST]; Cell Biloabs, INC. USA) used for the assay were prepared in the following manner: the 100 µl sperm sample was diluted in 5 ml of PBS (phosphate buffer solution; Ca^{2+} and Mg^{2+} free) to obtain a spermatozoa density of 1×10^7 cells/ml. Diluted samples (200 µl) were mixed with 700 µl 0.8% NuSieve GTG low melting point agarose (OxiSelect[ST]; Cell Biloabs, INC. USA). Finally, 55 µl of this mixture was added to the slide, and the agarose was allowed to solidify for 1 h at 4 °C. The slides were immersed in lysis buffer (2.5 M NaCl, 100 mM EDTA, 10% lysis Solution [OxiSelect™ Comet Assay Kit], 10% DMSO, pH 10) at 4 °C. Slides were then subjected to enzyme treatment with proteinase K (1 mg/ml in 2.5 M NaCl, 100 mM EDTA, 10% DMSO, pH 7.4) and incubated overnight at 32 °C. After the proteinase K solution was drained, slides were immersed in a horizontal gel tank filled with freshly mixed electrophoresis buffer (90 mM Tris Base, 90 mM Boric acid, 2.5 mM EDTA). Electrophoresis was carried out for 20 min at 35 V and 170 mA. Slides were carefully transferred from the electrophoresis chamber to a clean container with pre-chilled DI H_2O for 2 min. The washing procedure was repeated three times. Slides were drained well, dehydrated by dipping into absolute ethanol for 5 min, and air-dried for storage. Prior to the analysis, 50 µl of Vista Green DNA Staining Solution (OxiSelect[ST]; Cell Biloabs, INC. USA) was added to each agarose spot on the slide and analyzed using an Olympus BX50 fluorescence microscope at 20× magnification. One hundred spermatozoa were scored for each sample, and the captured images were analyzed using CometScore image analysis software (TriTek Corporation, USA). Tail length (measured from the middle of the head to the end of the comet tail) and % Tail DNA (content of DNA in comet tail) were measured. The percent DNA in tail and Olive tail moment were calculated using the following formulas:

$$\%Tail\ DNA = 100 \times Tail\ DNA\ intensity/Cell\ DNA\ intensity$$

$$Olive\ tail\ moment = \%Tail\ DNA \times tail\ moment\ length.$$

2.6. Oxidative stress indices

Sperm samples were centrifuged at 5000×g at 4 °C for 10 min. The supernatant was carefully collected and discarded. The spermatozoa pellet was diluted with 50 mM potassium phosphate (KPi) buffer, pH 7.0, containing 0.5 mM EDTA to obtain a spermatozoa concentration of 5×10^8 cells/ml, then homogenized in an ice bath using a Sonopuls HD 2070 ultrasonicator (Bandelin Electronic,

Influence of environmentally relevant concentrations of vinclozolin on quality, DNA integrity, and antioxidant responses of sterlet Acipenser ruthenus spermatozoa

Berlin, Germany). The homogenate was divided into two portions, one for measuring thiobarbituric acid reactive substances (TBARS) and carbonyl derivatives of proteins (CP) and a second that was centrifuged at $12,000 \times g$ for 30 min at 4 °C to obtain the post mitochondrial supernatant for the antioxidant enzyme activity assay.

The TBARS method described by Lushchak et al. [20] was used to evaluate sperm lipid oxidation (LO). Its concentration was calculated by absorption at 535 nm and a molar extinction coefficient of 156 mM/cm. The content of TBARS was expressed as nanomoles per 10^8 cells. Carbonyl derivatives of proteins were detected by reaction with 2,4-dinitrophenylhydrazine according to the method described by Lenz et al. [21]. The amount of CP was measured spectrophotometrically at 370 nm using a molar extinction coefficient of 22 mM/cm, expressed as nmol per 10^8 cells.

2.7. Superoxide dismutase activity

Total SOD (EC 1.15.1.1) activity was determined by the method of Marklund and Marklund [22]. This assay depends on the autoxidation of pyrogallol. The activity of SOD in supernatant was assessed spectrophotometrically at 420 nm. One unit of SOD activity is defined as the amount of the enzyme needed to exhibit 50% dismutation of the superoxide radical per min. Activity was expressed in international units (or milliunits) mU per 10^8 cells.

2.8. Evaluation of ATP content

ATP content in spermatozoa was determined after 2 h exposure to VIN using the bioluminescence method described by Boryshpolets et al. [23]. Sperm samples were diluted in 5 ml boiling medium containing 100 mM Tris–HCl, pH 7.5 and 4 mM EDTA. After boiling for 2 min at 98 °C samples were centrifuged at $12,000 \times g$ for 20 min. Supernatant was collected and stored at −80 °C before analysis. A Bioluminescence Assay Kit CLS II (Roche Diagnostics GmbH, Germany) was used for determination of ATP content. Luminescence was recorded with a multifunctional microplate reader Infinite M200 (Tecan, Austria). ATP content was expressed as nmol ATP/10^8 spermatozoa.

2.9. Data analysis

All measurements were conducted in triplicate. Normality and the homogeneity of variance of all data were first tested with the Kolmogorov test and the Bartlett test, respectively. Data obtained from the Comet assay were log-transformed if necessary. Values are expressed as means ± SD ($n = 6$) and analyzed by factorial ANOVA. Statistical comparison was made by analysis of variance (ANOVA) followed by Tukey's HSD test for each analyzed parameter. The relationships among the parameters were quantified according to the Spearman's correlation tests. Values for motility and velocity only at 10 s post-activation were used. The t-test was used to establish if the correlation coefficients are significantly different from zero. All analyses were performed at a significance level of 0.05 using STATISTICA 9.0 software for Windows.

3. Results

3.1. Spermatozoa motility and velocity

The effect of VIN on percent motile spermatozoa and their curvilinear velocity was assessed 10, 30, 60, 90, and 120 s post-activation (Fig. 1 (a and b)). Motility and velocity significantly lower than in the control were observed after exposure to concentrations of VIN from 2 to 50 μg/l ($P < 0.05$) at all post-activation times. Motility parameters of spermatozoa exposed to the lowest concentration of VIN (0.5 μg/l) were significantly different from the control only at 10 and 30 s post-activation. In general, motility and velocity of spermatozoa exposed to VIN concentrations of 15–50 μg/l were 16–20% lower that in control. A significant positive correlation ($r = 0.713$, $P < 0.05$) was found between percent motile spermatozoa and ATP content (Table 1). A significant negative correlation ($r = −0.628$, $P < 0.05$) between spermatozoa motility and the level of protein carbonylation was observed. Fig. 2 illustrates changes in flagella movement after exposure of spermatozoa to VIN. The beat frequency and number of waves along the flagellum were significantly lower at the highest concentration of VIN compared to control. Atypical C shaped flagella were observed.

3.2. DNA fragmentation

An illustration of the outcome of this assay for DNA damage is presented in Fig. 3. Obtained pictures of comets were used to evaluate following parameters:% of DNA in Tail (calculated by formula:% Tail DNA = $100 \times$ Tail DNA Intensity/Cell DNA Intensity) and Olive tail moment (Olive tail moment = % tail DNA × tail moment length). Comet assay analysis indicated a dramatic increase in DNA fragmentation in spermatozoa nuclei after 2 h exposure to VIN at concentrations of 10–50 μg/l compared to controls ($P < 0.05$; Fig. 4(a and b). No significant differences ($P > 0.05$) in DNA integrity were found between the control group and spermatozoa exposed to nominal VIN concentrations of 0.5–2 μg/l. The highest VIN concentration was associated with 20% DNA fragmentation (Fig. 4a). There was significant correlation between% DNA in tail and TBARS level (Table 1) ($r = 0.354$, $P < 0.05$).

3.3. Oxidative stress indices and antioxidant response

The level of TBARS and CP were used as indicators of the extent of LO and protein oxidation, respectively. In the control group, TBARS level was typically around 0.16 nmol/10^8 spermatozoa (Fig. 5). A significantly higher level of LO was observed in spermatozoa exposed to VIN at 10 μg/l and above. The highest concentration of VIN enhanced the TBARS level to 0.33 nmol/10^8 spermatozoa. There was no significant difference in CP level between spermatozoa in the control group and group exposed to 0.5 μg/l VIN (Fig. 6). A gradual increase in CP level was observed in groups exposed to concentrations greater than 2 μg/l. The maximum detected CP was 11.6 nmol/10^8 in spermatozoa exposed to 50 μg/l VIN. Spearman's correlation revealed significant negative correlation between ATP content and TBARS ($r = −0.66$, $P < 0.05$) and CP ($r = −0.61$, $P < 0.05$) levels (Table 1).

The antioxidant activity was assessed by total SOD activity. The antioxidant response was significantly enhanced in all treatment groups (Fig. 7). Total SOD was 6.5 mU/10^8 in spermatozoa at 50 μg/l of VIN compared to 2 mU/10^8 in controls. An increase in this parameter was not observed with increasing concentrations of VIN. A positive correlation was found between SOD activity and CP level ($r = 0.41$, $P < 0.05$).

3.4. ATP content

The ATP levels can indicate decline in quality of fish spermatozoa. Intracellular ATP content in the control group was 6.8 nmol ATP/10^8 spermatozoa (Fig. 8). After 2 h exposure to VIN at concentrations higher than 2 μg/l, the ATP level declined significantly in a dose-dependent manner. The lowest ATP content was approximately 5.0 nmol ATP/10^8 spermatozoa at VIN concentrations of 15–20 μg/l. ATP content in spermatozoa exhibited a significant positive correlation with motility ($r = 0.71$, $P < 0.05$) and velocity ($r = 0.29$, $P < 0.05$) and showed a significant
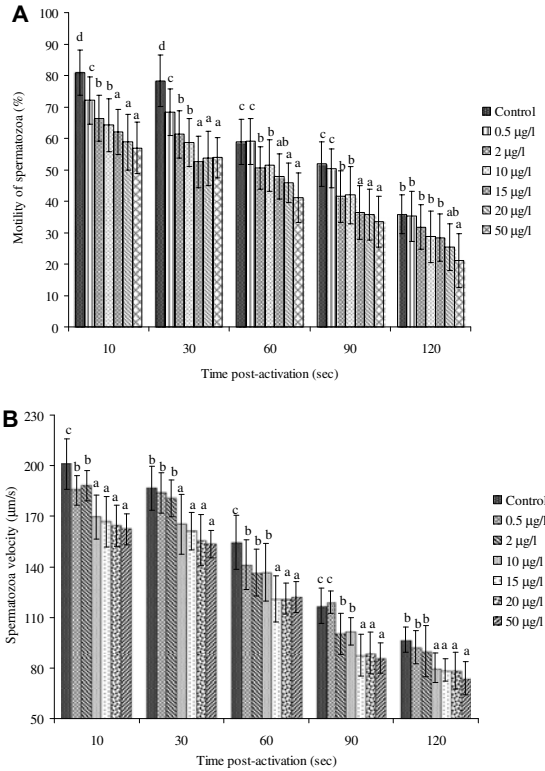
Fig. 1. *In vitro* effects of vinclozolin on spermatozoa motility (a) and velocity (b) in *Acipenser ruthenus*. Data are presented as means ± SD. Superscript letters indicate significant differences among samples at the same time post-activation (ANOVA, *P* < 0.05).

Table 1
Correlation coefficients among the parameters measured in *Acipenser ruthenus* spermatozoa after 2 h exposure to vinclozolin. The *t*-test is used to establish significance of the correlation between pairs of parameters.

	Motility	Velocity	%DNA in tail	Olive tail moment	TBARS	CP	SOD	ATP
Motility	1.00							
Velocity	0.28	1.00						
%DNA in tail	−0.36*	−0.19*	1.00					
Olive tail moment	−0.33*	−0.23*	0.92*	1.00				
TBARS	−0.43*	−0.202	0.35*	0.29*	1.00			
CP	−0.62*	−0.30*	0.15	0.14	0.24	1.00		
SOD	−0.45*	0.02	0.11	0.18	0.21	0.41*	1.00	
ATP	0.71*	0.29*	−0.27*	−0.2	−0.66*	−0.61*	−0.28*	1.00

* *P* < 0.05 according to the Spearman's test.

negative correlation with TBARS level (*r* = −0.66, *P* < 0.05) and CP (*r* = −0.61, *P* < 0.05) (Table 1).

4. Discussion

Environmental contaminants have been shown to induce reproductive dysfunction in both wildlife and humans [12,24]. Despite the volume of research into the effects of chemicals having endocrine-disrupting properties in fish, there is limited evidence that endocrine disruption adversely affects individual fertility and, hence, poses problems for the wider population. In this study an attempt was made to determine *in vitro* the effects of anti-androgen VIN on spermatozoa performance, using sterlet as a model organism. Results of the present study suggest that sturgeon spermatozoa quality, DNA integrity, ATP metabolism, and the antioxidant defense system can be impaired by VIN-mediated oxidative stress.

Because variations in spermatozoa motility might reflect quantitative and qualitative toxic effects of ectogenous contaminants,
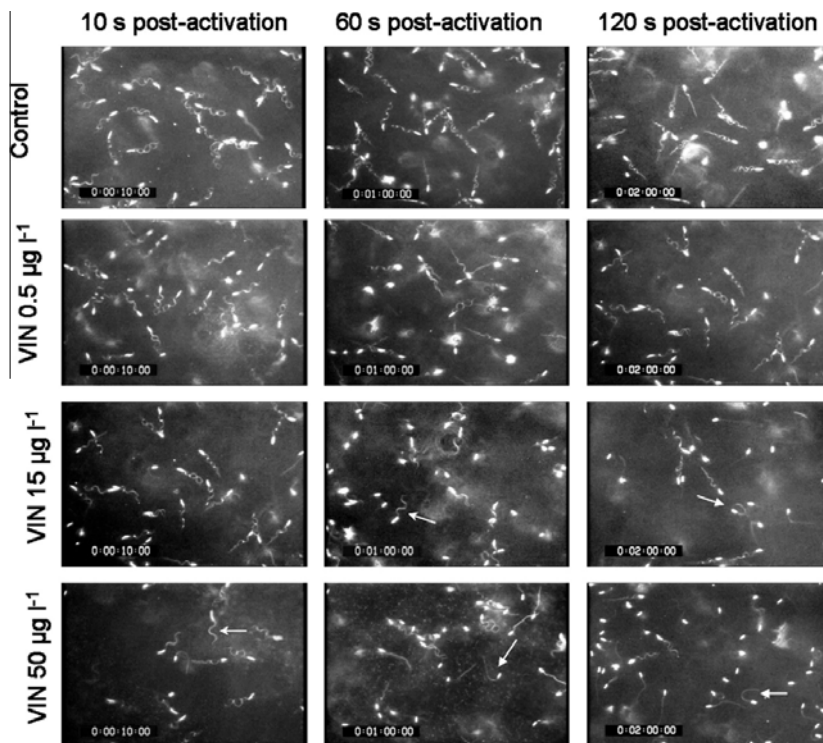
Influence of environmentally relevant concentrations of vinclozolin on quality, DNA integrity, and antioxidant responses of sterlet Acipenser ruthenus spermatozoa

Fig. 2. Swimming behavior of *Acipenser ruthenus* spermatozoa incubated with three concentrations of vinclozolin (VIN) (0.5, 15, 50 µg/l). Flagella movement was recorded under dark field microscopy with stroboscopic illumination at 10, 60, and 120 s post-activation of spermatozoa. Arrows indicate various forms of flagella detected after sperm exposure to VIN *in vitro*.

monitoring of spermatozoa motility parameters is an important approach in toxicity experiments [25]. In the current study, spermatozoa motility and velocity were significantly reduced after *in vitro* exposure to environmentally relevant concentrations of VIN. There are several possible ways in which anti-androgens may affect spermatozoa motility activation and movement. In mammals, it has been shown that anti-androgens repress androgen receptor (AR) mediated transcriptional activation, by competitive inhibition of endogenous androgens binding to their receptor [26–30]. Binding of anti-androgens may result in a conformational change of the ligand binding domain of AR to bring about interaction with co-repressors instead of co-activators [31,32]. Besides classical intracellular steroid hormone receptors, several membrane steroid receptors, capable of mediating non-genomic steroid actions, have been described [33,34]. Despite the limited amount of information about membrane receptors in fish spermatozoa, it is noteworthy that Thomas and Doughty [35] observed that non-estrogenic as well as estrogenic organic compounds could interfere with a rapid non-genomic progestin action to up-regulate spermatozoa motility in Atlantic croaker (*Micropogonias undulatus*).

ATP metabolism is another potential target of VIN. ATP is essential for spermatozoa motility, and decreased ATP equates with decreased spermatozoa movement [36]. Several studies have shown that aquatic pollution might result in intracellular ATP depletion within cells [4,36]. Pathways to inhibition of ATP

production may differ with pollutant. In general, anti-androgen agents inhibit the mitochondrial respiratory chain and ATP formation [37]. In the present study, the percent of motile spermatozoa was strongly correlated with intracellular ATP content. Both parameters decreased after 2 h incubation with VIN. ATP concentration within the cell determines motility duration and flagella beat frequency [36,38]. Therefore, decrease of ATP content could explain changes in flagella behavior observed in the present study.

Studies have demonstrated that VIN can induce production of ROS in cell cultures [39,40]. Oxidative stress results in direct or indirect ROS-mediated damage of nucleic acids, proteins, and lipids. The toxic effect of ROS on fish spermatozoa has been reported for several species [41,42]. In mammals it has been shown that an accumulation of peroxidation products in mitochondria leads to a decrease in ATP production and compromises the maintenance of cell homeostasis [43]. In our study a significant correlation was found between ATP content and the level of TBARS and CP. Therefore, we hypothesize that the accumulation of free radicals (LO and CP) in spermatozoa could indirectly modify energy metabolism.

Increased levels of LO and CP observed in the present study also suggest spermatozoa susceptibility to VIN-induced free radical production. TBARS levels were greater in spermatozoa incubated with VIN at concentrations >10 µg/l. This is not surprising, since
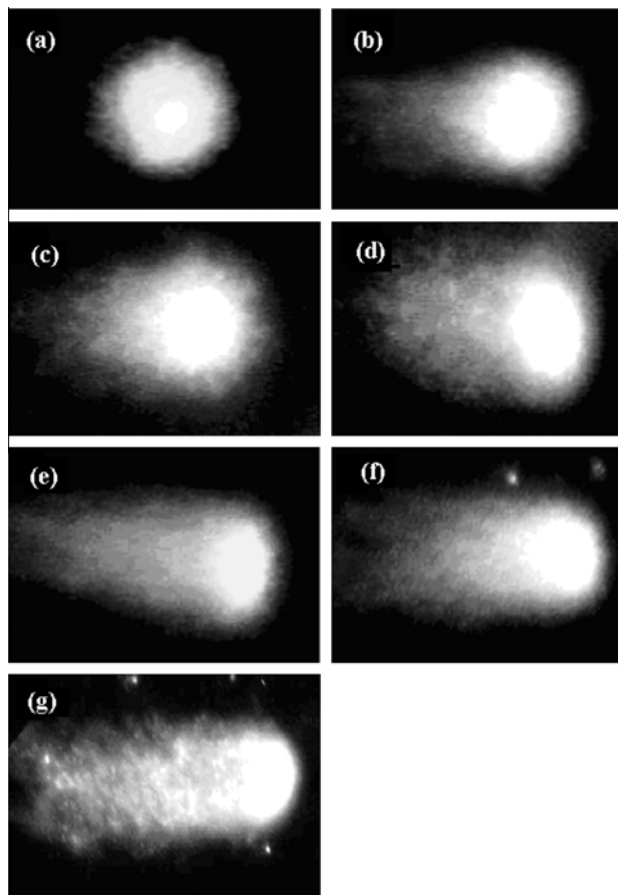
Fig. 3. Examples from Comet assay of *Acipenser ruthenus* spermatozoa exposed to differing concentrations of vinclozolin (a) control, (b) 0.5 µg/l, (c) 2 µg/l, (d) 10 µg/l, (e) 15 µg/l, (f) 20 µg/l, and (g) 50 µg/l). Relative changes in DNA fragmentation are represented by an increasing amount of DNA in the comet tail.

a major mechanism of action of VIN is lipid peroxidation and membrane disruption [44]. Peroxidation of PUFAs in spermatozoa cell membranes is an autocatalytic, self-propagating reaction, which can give rise to cell dysfunction [11]. Lipid oxidation can trigger the loss of membrane integrity, causing increased cell permeability, enzyme inactivation, and structural damages of DNA [45,46].

Increase in CP level also indicates oxidative stress in the cell. Protein carbonylation is the most frequently used marker of protein oxidation. There is evidence that oxidative modification leads to proteolytic degradation, which may affect the structure, function, and integrity of proteins [41,47,48]. Previous studies have shown that protein oxidation and accumulation of lipid hydroperoxides in the plasma membrane can profoundly affect fertilization ability of spermatozoa [48]. In this study we observed increased carbonylation of proteins after 2 h exposure to concentrations of VIN higher than 2 µg/l. This demonstrates that environmentally realistic concentrations of anti-androgens such us VIN are capable

of inducing protein oxidation as well as lipid oxidation in sterlet spermatozoa.

To counteract the damaging effects of ROS, a variety of antioxidant enzymes are present in fish spermatozoa [41,49]. Superoxide dismutase is considered the first line of defense against the effects of oxyradicals in the cell through catalyzing the dismutation of superoxide radicals to hydrogen peroxide and molecular oxygen [5]. In the present study, significantly increased SOD activity was observed in all treatment groups. This is likely to be an adaptive response to toxicant stress and serves to neutralize the impact of increased ROS generation.

Studies of human spermatozoa have revealed that oxidative stress may induce significant DNA damage in both the mitochondrial and nuclear genomes [50]. Lack of repair mechanisms renders spermatozoan DNA vulnerable to damage, and this damage is linked to male infertility [51]. Vinclozolin is known to cause DNA methylation in germ cells of rats and mice and to promote
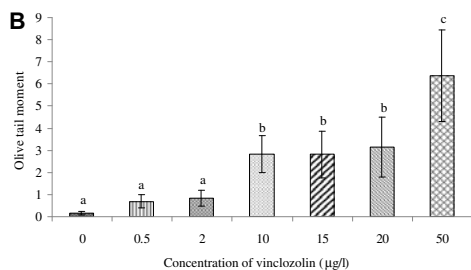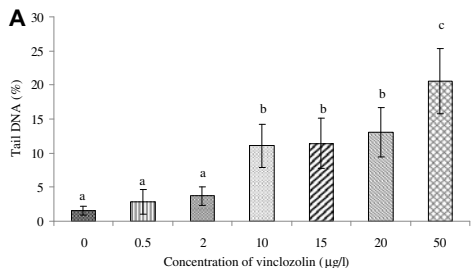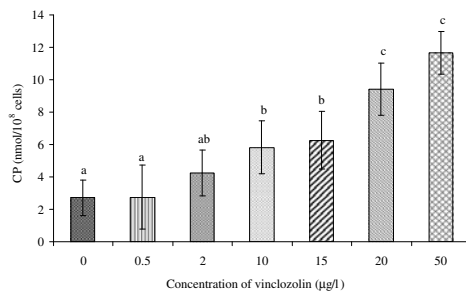
Fig. 4. Influence of vinclozolin on DNA fragmentation in *Acipenser ruthenus* spermatozoa measured by Comet assay as a percentage of DNA in the tail (a) and Olive tail moment (b). Data represent mean values ± SD. Different letters indicate significant differences among samples (ANOVA, $P < 0.05$).
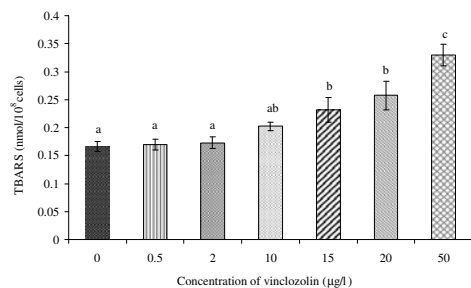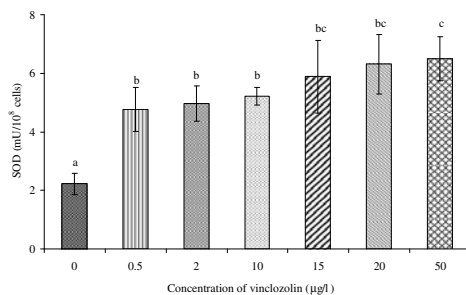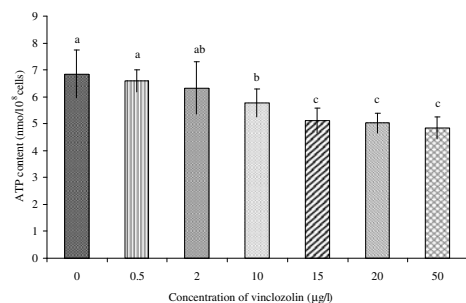

Fig. 5. Effect of vinclozolin on TBARS level in *Acipenser ruthenus* spermatozoa. Data represent mean values ± SD. Different letters indicate significant differences among samples (ANOVA, $P < 0.05$).

Fig. 6. Effect of vinclozolin on CP level in *Acipenser ruthenus* spermatozoa. Data represent mean values ± SD. Different letters indicate significant differences among samples (ANOVA, $P < 0.05$).

Fig. 7. Effect of vinclozolin on SOD activity in *Acipenser ruthenus* spermatozoa. Data represent mean values ± SD. Different letters indicate significant differences among samples (ANOVA, $P < 0.05$).

Fig. 8. Influence of vinclozolin on ATP content in *Acipenser ruthenus* spermatozoa. Data represent mean values ± SD. Different letters indicate significant differences among samples (ANOVA, $P < 0.05$).

transgenerational disease [52]. However, no data have been reported on the effect of VIN on DNA integrity of spermatozoa of aquatic organisms. In the present study, the Comet assay was used to evaluate the effect of xenobiotic VIN on DNA integrity. Exposure to VIN at concentrations higher than 10 μg/l enhanced DNA fragmentation, expressed as percent of DNA in comet tail and Olive tail moment. Positive correlation was found between the level of DNA fragmentation and oxidative stress indices, indicating that the observed DNA damage was a result of a free radical production. Correlation between loss of DNA integrity and increased TBARS level is in accordance with other reports [53,54], indicating that lipid oxidation results in the production of reactive products which are a major source of endogenous DNA damage.

Results of the present study demonstrated that sterlet spermatozoa are highly sensitive to the presence of small concentrations of anti-androgenic compounds such as VIN. Environmentally relevant concentrations of this fungicide are able to induce oxidative stress in spermatozoa leading to reduced motility,

DNA integrity, and ATP content. Though the antioxidant system apparently responds to increased lipid oxidation and carbonyl-ation of proteins, it seems that the antioxidant capacity of spermatozoa is not sufficient to prevent cell damage.

Conflict of interest statement

No conflict of interest exists.

References

[1] N. Martí, D. Aguado, L. Segovia-Martínez, A. Bouzas, A. Seco, Occurrence of priority pollutants in WWTP effluents and Mediterranean coastal waters of Spain, Mar. Pollut. Bull. 62 (2011) 615–625.

[2] D.E. Kime, J.P. Nash, Gamete viability as an indicator of reproductive endocrine disruption in fish, Sci. Total Environ. 233 (1999) 123–129.

[3] A. Arukwe, Cellular and molecular responses to endocrine-modulators and the impact on fish reproduction, Mar. Pollut. Bull. 42 (2001) 643–655.

[4] E. Rurangwa, A. Biegniewska, E. Slominska, E.F. Skorkowski, F. Ollevier, Effect of tributyltin on adenylate content and enzyme activities of teleost sperm: a biochemical approach to study the mechanisms of toxicant reduced spermatozoa motility, Comp. Biochem. Physiol. C: Toxicol. Pharmacol. 131 (2002) 335–344.

[5] Z.H. Li, P. Li, B. Dzyuba, T. Randak, Influence of environmental related concentrations of heavy metals on motility parameters and antioxidant responses in sturgeon sperm, Chem. Biol. Interact. 188 (2010) 473–477.

[6] S. Brooks, C.R. Tyler, J.P. Sumpter, Quality in fish: what makes a good egg?, Rev Fish. Biol. Fish. 7 (1997) 387–416.

[7] V.J. Birstein, W.E. Bemis, J.R. Waldman, The threatened status of acipenseriform species: a summary, Environ. Biol. Fishes. 48 (1997) 427–435.

[8] M.J.G. Gage, C.P. Macfarlane, S. Yeates, R.G. Ward, J.B. Searle, G.A. Parker, Spermatozoal traits and sperm competition in Atlantic salmon: relative sperm velocity is the primary determinant of fertilization success, Curr. Biol. 14 (2004) 44–47.

[9] V. Kaspar, K. Kohlmann, M. Vandeputte, M. Rodina, D. Gela, M. Kocour, S.M.H. Alavi, M. Hulak, O. Linhart, Equalizing sperm concentrations in a common carp (*Cyprinus carpio*) sperm pool does not affect variance in proportions of larvae sired in competition, Aquaculture 272 (Suppl. 1) (2007) S204–S209.

[10] V. Kaspar, M. Hulak, K. Kohlmann, M. Vandeputte, M. Rodina, D. Gela, O. Linhart, In vitro study on sperm competition in common carp (*Cyprinus carpio* L.), Cybium 32 (2008) 303–306.

[11] D. Sanocka, M. Kurpisz, Reactive oxygen species and sperm cells, Reprod. Biol. Endocrinol. 2 (2004) 12.

[12] B.S. Zhou, W.H. Liu, W.H.L. Siu, D. O'Toole, P.K.S. Lam, R.S.S. Wu, Exposure of spermatozoa to duroquinone may impair reproduction of the common carp (*Cyprinus carpio*) through oxidative stress, Aquat. Toxicol. 77 (2006) 136–142.

[13] D.A. Lambropoulou, T.A. Albanis, Sensitive trace enrichment of environmental andiandrogen vinclozolin from natural waters and sediment samples using hollow-fiber liquid-phase microextraction, J. Chromatogr. A 1061 (2004) 11–18.

[14] T. Steeger, K. Garber, Risks of vinclozolin use to federally threatened California red-legged frog (*Rana aurora draytonii*), Environmental Fate and Effects Division, Office of Pesticide Programs, Washington, DC, 20460, 2009.

[15] S.Y. Szeto, N.E. Burlinson, J.E. Rahe, P.C. Oloffs, Kinetics of hydrolysis of the dicarboximide fungicide vinclozolin, J. Agric. Food Chem. 37 (2) (1989) 523–529.

[16] C.I. Wong, W.R. Kelce, M. Sar, E.M. Wilson, Androgen receptor antagonist versus agonist activities of the fungicide vinclozolin relative to hydroxyflutamide, J. Biol. Chem. 270 (1995) 19998–20003.

[17] E. Baatrup, M. Junge, Antiandrogenic pesticides disrupt sexual characteristics in the adult male guppy (*Poecilia reticulata*), Environ. Health Perspect. 109 (2001) 1063–1070.

[18] M. Bayley, M. Junge, E. Baatrup, Exposure of juvenile guppies to three antiandrogens causes demasculinization and a reduced sperm count in adult males, Aquat. Toxicol. 56 (2002) 227–239.

[19] P. Li, Q. Wei, L. Liu, DNA integrity of *Polyodon spathula* cryopreserved sperm, J. Appl. Ichthyol. 24 (2008) 121–125.

[20] V.I. Lushchak, T.V. Bagnyukova, V.V. Husak, L.I. Luzhna, O.V. Lushchak, K.B. Storey, Hyperoxia results in transient oxidative stress and an adaptive response by antioxidant enzymes in goldfish tissues, Int. J. Biochem. Cell. Biol. 37 (2005) 1670–1680.

[21] A.G. Lenz, U. Costabel, S. Shaltiel, R.L. Levine, Determination of carbonyl groups in oxidatively modified proteins by reduction with tritiated sodium-borohydride, Anal. Biochem. 177 (1989) 419–425.

[22] S. Marklund, G. Marklund, Involvement of the superoxide anion radical in the autoxidation of pyrogallol and a convenient assay for superoxide dismutase, Eur. J. Biochem. 47 (1974) 469–474.

[23] S. Boryshpolets, B. Dzyuba, M. Rodina, P. Li, M. Hulak, D. Gela, O. Linhart, Freeze-thawing as the factor of spontaneous activation of spermatozoa motility in common carp (*Cyprinus carpio* L.), Cryobiology 59 (2009) 291–296.

[24] G. Grizard, L. Ouchchane, H. Roddier, C. Artonne, B. Sion, M.P. Vasson, L. Janny, In vitro alachlor effects on reactive oxygen species generation, motility patterns and apoptosis markers in human spermatozoa, Reprod. Toxicol. 23 (2007) 55–62.

[25] E. Rurangwa, D.E. Kime, F. Ollevier, J.P. Nash, The measurement of sperm motility and factors affecting sperm quality in cultured fish, Aquaculture 234 (2004) 1–28.

[26] C. Wolf, C. Lambright, P. Mann, M. Price, R.L. Cooper, J. Ostby, L.E. Jr, Gray, Administration of potentially antiandrogenic pesticides (procymidone, linuron, iprodione, chlozolinate, p, p′-DDE, and ketoconazole) and toxic substances (dibutyl- and diethylhexyl phthalate, PCB 169, and ethane dimethane sulphonate) during sexual differentiation produces diverse profiles of reproductive malformations in the male rat, Toxicol. Ind. Health 15 (1999) 94–118.

[27] C. Lambright, J. Ostby, K. Bobseine, V. Wilson, A.K. Hotchkiss, P.C. Mann, L.E. Jr, Gray, cellular and molecular mechanisms of action of linuron: an antiandrogenic herbicide that produces reproductive malformations in male rats, Toxicol. Sci. 56 (2000) 389–399.

[28] A.M. Vinggaard, C. Nellemann, M. Dalgaard, E.B. Jorgensen, H.R. Andersen, Antiandrogenic effects in vitro and in vivo of the fungicide prochloraz, Toxicol. Sci. 69 (2002) 344–353.

[29] N.C. Noriega, J. Ostby, C. Lambright, V.S. Wilson, L.E. Gray, Late gestational exposure to the fungicide prochloraz delays the onset of parturition and causes reproductive malformations in male but not female rat offspring, Biol. Reprod. 72 (2005) 1324–1335.

[30] M.L. Mohler, C.E. Bohl, A. Jones, C.C. Coss, R. Narayanan, Y.L. He, D.J. Hwang, J.T. Dalton, D.D. Miller, Nonsteroidal selective androgen receptor modulators (SARMs): dissociating the anabolic and androgenic activities of the androgen receptor for therapeutic benefit, J. Med. Chem. 52 (2009) 3597–3617.

[31] C.A. Berrevoets, A. Umar, A.O. Brinkmann, Antiandrogens: selective androgen receptor modulators, Mol. Cell. Endocrinol. 198 (2002) 97–103.

[32] M.C. Hodgson, H.C. Shen, A.N. Hollenberg, S.P. Balk, Structural basis for nuclear receptor corepressor recruitment by antagonist-liganded androgen receptor, Mol. Cancer Ther. 7 (2008) 3187–3194.

[33] P. Thomas, J. Dong, Binding and activation of the seven-transmembrane estrogen receptor GPR30 by environmental estrogens: a potential novel mechanism of endocrine disruption, J. Steroid Biochem. Mol. Biol. 102 (2006) 175–179.

[34] C.S. Watson, N.N. Bulayeva, A.L. Wozniak, C.C. Finnerty, Signaling from the membrane via membrane estrogen receptor-alpha: estrogens, xenoestrogens, and phytoestrogens, Steroids 70 (2005) 364–371.

[35] P. Thomas, K. Doughty, Disruption of rapid, nongenomic steroid actions by environmental chemicals: interference with progestin stimulation of sperm motility in Atlantic croaker, Environ. Sci. Technol. 38 (2004) 6328–6332.

[36] G. Perchec, C. Jeulin, J. Cosson, F. Andre, R. Billard, Relationship between sperm ATP content and motility of carp spermatozoa, J. Cell Sci. 108 (1995) 747–753.

[37] A. Berson, I. Schmets, C. Fisch, D. Fau, C. Wolf, B. Fromenty, D. Deschamps, D. Pessayre, Inhibition by nilutamide of the mitochondrial respiratory chain and ATP formation. Possible contribution to the adverse effects of this antiandrogen, J. Pharmacol. Exp. Ther. 270 (1994) 167–176.

[38] S.M.H. Alavi, Fish Spermatology, Alpha Science International Ltd, 2008.

[39] S. Radice, L. Marabini, M. Gervasoni, M. Ferraris, E. Chiesara, Adaptation to oxidative stress: effects of vinclozolin and iprodione on the HepG2 cell line, Toxicology 129 (1998) 183–191.

[40] S.M.J.C.S. Cabral, J.P.S. Cabral, The primary mode-of-action of vinclozol: are oxygen free radicals directly involved?, Pestic Biochem. Physiol. 66 (2000) 145–152.

[41] P. Li, Z.H. Li, B. Dzyuba, M. Hulak, M. Rodina, O. Linhart, Evaluating the impacts of osmotic and oxidative stress on common carp (*Cyprinus carpio*, L.) sperm caused by cryopreservation techniques, Biol. Reprod. 83 (2010) 852–858.

[42] G.J. Dietrich, A. Szpyrka, M. Wojtczak, S. Dobosz, K. Goryczko, L. Zakowski, A. Ciereszko, Effects of UV irradiation and hydrogen peroxide on DNA fragmentation, motility and fertilizing ability of rainbow trout (*Oncorhynchus mykiss*) spermatozoa, Theriogenology 64 (2005) 1809–1822.

[43] B. Chance, H. Sies, A. Boveris, Hydroperoxide metabolism in mammalian organs, Physiol. Rev. 59 (1979) 527–605.

[44] G.J. Choi, H.J. Lee, K.Y. Cho, Lipid peroxidation and membrane disruption by vinclozolin in dicarboximide-susceptible and -resistant isolates of *Botrytis cinerea*, Pestic. Biochem. Physiol. 55 (1996) 29–39.

[45] R.J. Aitken, D. Buckingham, D. Harkiss, Use of a xanthine-oxidase free-radical generating-system to investigate the cytotoxic effects of reactive oxygen species on human spermatozoa, J. Reprod. Fertil. 97 (1993) 441–450.

[46] M.J. McCarthy, J. Baumber, P.H. Kass, S.A. Meyers, Osmotic stress induces oxidative cell damage to rhesus macaque spermatozoa, Biol. Reprod. 82 (2010) 644–651.

[47] B.C. Almroth, J. Sturve, E. Stephensen, T.F. Holth, L. Forlin, Protein carbonyls and antioxidant defenses in corkwing wrasse (*Symphodus melops*) from a heavy metal polluted and a PAH polluted site, Mar. Environ. Res. 66 (2008) 271–277.

[48] S.B. DuTeaux, T. Berger, R.A. Hess, B.L. Sartini, M.G. Miller, Male reproductive toxicity of trichloroethylene: sperm protein oxidation and decreased fertilizing ability, Biol. Reprod. 70 (2004) 1518–1526.

[49] P. Li, M. Hulak, O. Linhart, Sperm proteins in teleostean and chondrostean (sturgeon) fishes, Fish Physiol. Biochem. 35 (2009) 567–581.

[50] R.J. Aitken, M.A. Baker, Oxidative stress, sperm survival and fertility control, Mol. Cell. Endocrinol. 250 (2006) 66–69.

[51] J. O'Brien, A. Zini, Sperm DNA integrity and male infertility, Urology 65 (2005) 16–22.

[52] M.D. Anway, Epigenetic transgenerational actions of endocrine disruptors and male fertility, Science 308 (2005) 1466–1469.

[53] U. Cobanoglu, H. Demir, A. Cebi, F. Sayir, H.H. Alp, Z. Akan, T. Gur, E. Bakan, Lipid peroxidation, DNA damage and CoenzymeQ10 in lung cancer patients – markers for risk assessment?, Asian Pac J. Cancer Prev. 12 (2011) 1399–1403.

[54] L.J. Marnett, Oxy radicals, lipid peroxidation and DNA damage, Toxicology 181 (2002) 219–222.

CHAPTER 3

PROTECTION OF COMMON CARP (*CYPRINUS CARPIO* L.) SPERMATOZOA MOTILITY UNDER OXIDATIVE STRESS BY ANTIOXIDANTS AND SEMINAL PLASMA

It was allowed by publisher on 22nd April 2015 to include the paper in this Ph.D. thesis.

Protection of common carp (*Cyprinus carpio* L.) spermatozoa motility under oxidative stress by antioxidants and seminal plasma

A. Shaliutina-Kolešová · I. Gazo · J. Cosson · O. Linhart

Abstract The protective influence of seminal plasma and the antioxidants catalase (CAT), superoxide dismutase (SOD), and glutathione (GTH) on quality parameters, oxidative stress indices, and antioxidant activity was studied in common carp (*Cyprinus carpio*) spermatozoa exposed to the xanthine–xanthine oxidase (X–XO) system. Fish spermatozoa were incubated for 5 and 20 min at 4 °C with X–XO concentrations of 1 mM X–0.1 U/mL, 0.6 mM X–0.05 U/mL, 0.3 mM X–0.025 U/mL, and 0.1 mM X–0.0125 U/mL. A dose-dependent reduction in spermatozoa motility and velocity was observed at concentrations of 0.1 mM X–0.0125 U/mL to 1 mM X–0.1 U/mL XO. Increase in spermatozoa motility parameters was recorded following treatment with antioxidants and seminal plasma. The level of the oxidative stress indices lipid peroxidation (LPO) and carbonyl derivatives of proteins (CP) was significantly reduced after addition of CAT, SOD, or GTH along with seminal plasma. Significant differences in SOD, glutathione reductase, and glutathione peroxidase activity were seen in spermatozoa incubated with, compared to that without, seminal plasma at all studied X–XO concentrations. The data demonstrated that CAT, SOD, or GTH in combination with SP can reduce reactive oxygen species stress in fish spermatozoa and improve spermatozoa quality.

Keywords Antioxidant enzymes · Carp sperm · Reactive oxygen species · Seminal plasma · Spermatozoa motility

Abbreviations

X–XO	Xanthine–xanthine oxidase
LPO	Lipid peroxidation
CP	Carbonyl proteins
TBARS	Thiobarbituric acid-reactive substances
SOD	Superoxide dismutase
GR	Glutathione reductase
GPx	Glutathione peroxidase

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Introduction

Spermatozoa, like all cells living in aerobic conditions, constantly face the oxygen paradox (de Lamirande and Gagnon 1995). Oxygen is essential for life, but its metabolites, such as reactive oxygen species (ROS), have detrimental effects on sperm characteristics (Baumber et al. 2000; Li et al. 2010). Studies indicate that high levels of ROS have potentially toxic impact on sperm quality and may alter cell function,

endanger cell survival, or both (Sikka 2001; Saleh and Agarwal 2002). However, a certain level of ROS is needed for normal sperm function (de Lamirande and Gagnon 1993). Spermatozoa damage may result from oxidative stress, the level of which depends on the balance between production and degradation of ROS (Sharma et al. 1999).

Fish spermatozoa are sensitive to damage by ROS, since they possess limited endogenous antioxidant protection while presenting abundant substrates for free radical attack on polyunsaturated fatty acids (Gazo et al. 2013; Koppers et al. 2010). When the production of ROS by spermatozoa mitochondria is excessive, the gamete's limited defenses are rapidly overwhelmed, and oxidative damage induces lipid peroxidation (LPO), with a resulting loss of vitality and fertilizing potential (Aitken et al. 1998). The loss of motility and velocity during short term in vitro storage in spermatozoa of Russian and Siberian sturgeon has been attributed to oxidative stress that significantly impaired cellular metabolism (e.g., oxidative phosphorylation), leading to a subsequent decline in motility (Shaliutina et al. 2013a, b).

Xanthine oxidase (XO) is one of several enzymes generating superoxide (O_2^-), and its mechanism of action has been thoroughly studied (Olson et al. 1974). Xanthine oxidase acting aerobically on xanthine generates O_2^- and hydrogen peroxide (H_2O_2). The xanthine–xanthine oxidase (X–XO) system is one of several ROS-generating systems frequently used in biology to evaluate the cytotoxic effects of O_2^- and H_2O_2. Generation of ROS in vitro by the X–XO system results in a reduction in spermatozoa motility (Hagedorn et al. 2012).

Semen contains an array of antioxidant mechanisms: Compounds and reactions that dispose of, scavenge, and suppress the formation of ROS or counteract their actions (Bansal and Bilaspuri 2010). Fish seminal plasma (SP) possesses a number of enzymatic and non-enzymatic components (Liu et al. 1995; Ciereszko et al. 2000; Lahnsteiner et al. 2010) that play a key role in sperm vitality. Non-enzymatic 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) have been shown to be important in defense of spermatozoa from oxidative attack, while superoxide dismutase (SOD), glutathione reductase (GR), catalase (CAT), and glutathione

peroxidase (GPx) (Li et al. 2010; Martínez-Páramo et al. 2012) constitute enzymatic antioxidants. Under in vitro storage conditions, semen antioxidant systems are slow and inefficient in protection of spermatozoa from ROS (Lahnsteiner et al. 2010). The addition of molecules with antioxidant capacity to freezing media provides effective protection against cold shock and reduces the oxidative damage during sperm storage (Cabrita et al. 2011).

We investigated the protective effects of the antioxidants CAT, SOD, and glutathione (GTH), along with SP of common carp (Cyprinus carpio) spermatozoa exposed to the X–XO system. The common carp is among the most abundantly cultured fish species and constitutes a representative model for teleostean species studies.

Materials and methods

All chemicals were purchased from the Sigma-Aldrich Co. (Praha, Czech Republic).

Maintenance of fish and sperm collection

Male common carp ($n = 6$), weighing 4.0–4.5 kg, were obtained from the experimental station of the Faculty of Fisheries and Protection of Waters, University of South Bohemia at Vodnany, Czech Republic. Prior to experimentation, fish were held in 4-m³ outdoor plastic tanks with a constant pond water flow rate of 0.2 L/s. The temperature was 18–21 °C, and O_2 was 6–7 mg L^{-1}. Spermiation was stimulated by intramuscular injection of carp pituitary powder dissolved in 0.9 % (w/v) NaCl solution at 2 mg kg^{-1} body weight 36 h prior to stripping. Sperm samples were obtained by abdominal massage and collected directly into 10-mL plastic syringes. Care was taken to avoid contamination with mucus, feces, or water. Samples were stored 30 min on ice (0–4 °C) in closed assay tubes until assessment.

Sperm sample preparation

Sperm samples from each of six males were separately centrifuged at 300g for 30 min at 4 °C. The supernatant was collected and stored on ice until use. Ten microliter of spermatozoa pellet was diluted in 990 μL of immobilizing medium (IM) (200 mM KCl, 30 mM Tris–HCl,

pH 8.0). Reactive oxygen species were generated using the X–XO system. The final volume of assay solutions was 1,000 μL. The samples were exposed for 5 and 20 min at 4 °C to the following treatments:

– control–10 μL of spermatozoa in 990 μL of IM with or without of SP
– 10 μL of spermatozoa plus 1 mM X–0.1 U/mL XO in 960 μL of IM
– 10 μL of spermatozoa plus 0.6 mM X–0.05 U/mL XO in 974 μL of IM
– 10 μL of spermatozoa plus 0.3 mM X–0.025 U/mL XO in 982 μL of IM
– 10 μL of spermatozoa plus 0.1 mM X–0.0125 U/mL XO in 986.5 μL of IM
– 10 μL of spermatozoa plus 1 mM X–0.1 U/mL XO in 960 μL of IM and 1 μL of SP
– 10 μL of spermatozoa plus 0.6 mM X–0.05 U/mL XO in 974 μL of IM and 1 μL of SP
– 10 μL of spermatozoa plus 0.3 mM X–0.025 U/mL XO in 982 μL of IM and 1 μL of SP
– 10 μL of spermatozoa plus 0.1 mM X–0.0125 U/mL XO in 986.5 μL of IM and 1 μL of SP

Two additional sets of spermatozoa samples were used to assess the effect of antioxidants. The first was exposed to 1 mM X–0.1 U/mL XO in IM combined with either CAT (Sigma-Aldrich Co., cat. no. C1345), SOD (Sigma-Aldrich Co., cat. no. S5395), or GTH (Sigma-Aldrich Co., cat. no. G6137). The remaining set was incubated with the same antioxidants (10 μL of 0.1 mM CAT, SOD, or GTH in 990 μL of assay solution) plus 1 μL of SP. Each sample was subdivided and incubated at 4 °C for either 5 or 20 min.

The spermatozoa concentration was calculated according to methods described by Caille et al. (2006). Spermatozoa concentration was expressed as 10^8 spermatozoa/mL of sperm.

Analysis of spermatozoa motility and velocity

Percent of motile sperm cells (%) and velocity of motile spermatozoa (μm s^{-1}) were assessed at 10 s post-activation. For triggering motility, 1 μL of sperm was diluted with 49 μL of distilled water on a glass slide pre-positioned on the microscope stage. To prevent spermatozoa from sticking to the slide, 0.1 % bovine serum albumin was added to the swimming medium. Spermatozoa motility was recorded with a CCD video camera (SONY SSCDC50AP, Japan)

mounted on a microscope illuminated with Exposure-Scope® (FROV, Czech Republic). Successive positions of spermatozoa heads were analyzed from video frames using Olympus Micro-Image software, Version 4.0.1. for Windows, with a special macro by Olympus C & S. The trajectory of the spermatozoa head movement was recorded and traced from five overlapping successive frames with frame 1 red, frames 2–4 green, and frame 5 blue. Spermatozoa motility and velocity were calculated as described by Rodina et al. (2007). Assessment of spermatozoa motility was conducted in triplicate for each sample.

Oxidative stress indices

Sperm samples were centrifuged at 5,000g at 4 °C for 10 min. The supernatant was carefully collected and discarded. The spermatozoa pellet was suspended in 50 mM potassium phosphate (KPi) buffer, pH 7.0, containing 0.5 mM EDTA to obtain a spermatozoa concentration of 5×10^8 cells/mL, and then 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 12,000g for 30 min at 4 °C 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 LPO in spermatozoa. The TBARS concentration was calculated by light absorption at 535 nm on a spectrophotometer, using a molar extinction coefficient of 156 mM cm^{-1}. The TBARS content was expressed as nanomoles per 10^8 cells.

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^{-1}, expressed as nmol per 10^8 cells. Oxidative stress indices were obtained in triplicate for each sample.

Antioxidant variables

Total SOD activity was determined by the method of Marklund and Marklund (1974), which depends on the

autoxidation of pyrogallol. The activity of SOD in the spermatozoa pellet was assessed spectrophotometrically at 420 nm. One unit of SOD activity was defined as the amount of the enzyme needed to exhibit 50 % dismutation of the superoxide radical per min. Activity was expressed in international units (or milliunits), mU per 10^8 cells.

Glutathione peroxidase activity was assayed based on the rate of NADPH oxidation at 340 nm by the coupled reaction with GR. The activity was determined using the extinction coefficient of 6.22×10^3 M/cm (Lawrence and Burk 1976) and expressed as international units (or milliunits), mU per 10^8 cells.

Glutathione reductase activity was estimated spectrophotometrically, measuring NADPH oxidation at 340 nm (Carlberg and Mannervik 1975). One unit of GPx or GR activity was 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 units (or milliunits), mU per 10^8 cells.

All antioxidant activity variables were obtained in triplicate for each sample.

Statistical analysis

Prior to statistical comparisons of the variables, data were tested by the Kolmogorov test and the Bartlett test. Percent motile spermatozoa and spermatozoa velocity were determined for each male at a single time point following activation. Measurements were conducted in triplicate. Values, including TBARS, CP, SOD, GR, and GPx level, were expressed as mean $\pm$ SD ($n = 6$) and analyzed by analysis of variance (factorial ANOVA). Statistical comparison was made by ANOVA followed by Tukey's HSD test for each analyzed variable. All analyses were performed at a significance level of 0.05 using STATISTICA 9.0 software for Windows.

Results

Spermatozoa motility and velocity

The X–XO system was effective in inducing a stress reaction in common carp. In the presence of X–XO system, motility and velocity of spermatozoa decreased in a time- and dose-dependent manner compared with the control.

Direct observation of undiluted sperm using dark-field microscopy showed most spermatozoa of common carp to be devoid of progressive motility, with the flagella straight and quivering slightly. Following dilution in activation medium, a majority of the samples showed 95–100 % spermatozoa motility within 10 s. During the incubation periods of 5 and 20 min, motility and velocity of spermatozoa decreased significantly (ANOVA; $P < 0.05$) with increasing levels of X–XO. Motility parameters of spermatozoa exposed to the highest concentration of xanthine (1 mM) and XO (0.1 U/mL) and incubated for 20 min showed the lowest values. The addition of CAT increased both spermatozoa velocity to 204.34 ± 7.87 μm s^{-1} after 5 min incubation and to 177.1 ± 10.7 μm s^{-1} after 20 min incubation as well as percentage of motile spermatozoa (to 90.4 ± 3.7 % after 5 min incubation and 85.6 ± 3.9 % after 20 min incubation). Moreover, SOD and GTH improved both the percentage of motile spermatozoa and spermatozoa curvilinear velocity (VCL) to near control levels (Figs. 1, 2). Significantly higher values of spermatozoa motility parameters (ANOVA; $P < 0.05$) were observed after incubation of spermatozoa with SP and X–XO at all tested concentrations compared with samples incubated without SP (Figs. 1, 2).

Oxidative stress indices

Levels of TBARS and CP were used as indicators of the extent of LPO and protein oxidation, respectively, to assess X–XO-generated oxidative stress in spermatozoa. Similar to spermatozoa motility and velocity, the level of LPO was affected by ROS in a dose- and time-dependent manner.

Significant differences (ANOVA; $P < 0.05$) in levels of TBARS were found between spermatozoa samples incubated with SP and samples incubated without SP at all analyzed X–XO concentrations (Fig. 3). The maximum level of LPO was observed in spermatozoa exposed to the X–XO system at concentrations of 1 mM–0.1 U/ml, with the minimum detected at 0.3 mM X–0.025U/mL XO in the presence of CAT and SP (0.18 ± 0.03 nmol/10^8 cells).

The level of CP increased significantly in spermatozoa of common carp with increasing incubation time in the presence of the X–XO system. However, there was no significant difference (ANOVA; $P > 0.05$) in CP between spermatozoa incubated with SP and that

Fig. 1 The xanthine–xanthine oxidase (X–XO) system and the impact of antioxidants and seminal plasma on common carp (*Cyprinus carpio*) spermatozoa velocity. *Control* indicates control group where sperm samples were incubated for 5 or 20 min in the IM (200 mM KCl, 30 mM Tris–HCl, pH 8.0) with or without of SP. Data are presented as mean ± SD. *Superscripts* indicate significant differences among samples (ANOVA, $P < 0.05$)
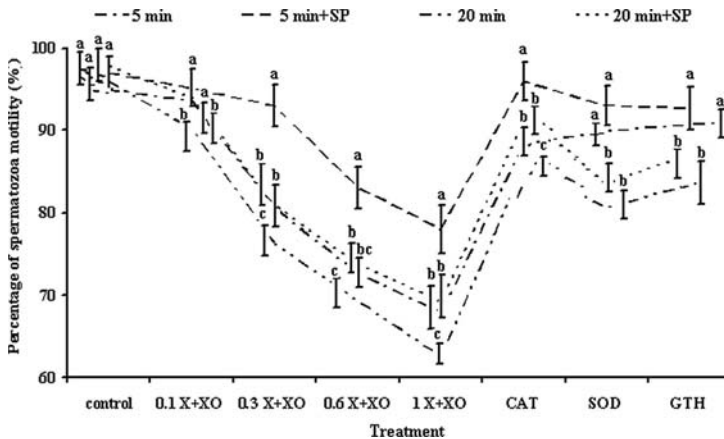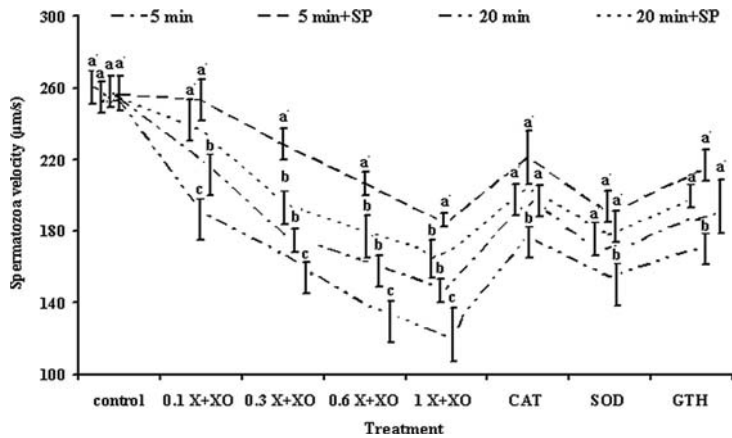
Fig. 2 The xanthine–xanthine oxidase (X–XO) system and the impact of antioxidants and seminal plasma on common carp (*Cyprinus carpio*) spermatozoa motility. *Control* indicates control group where sperm samples were incubated for 5 or 20 min in the IM (200 mM KCl, 30 mM Tris–HCl, pH 8.0) with or without of SP. Data are presented as mean ± SD. *Superscripts* indicate significant differences among samples (ANOVA, $P < 0.05$)

without over 5-min incubation. The highest detected CP level was 23.2 nmol/10^8 cells in spermatozoa exposed to 1 mM X–0.1 U/mL XO in IM, combined with SOD (Fig. 4).

Enzymatic responses

The antioxidant activity was assessed as GR, GPx, and total SOD activity. Significant changes (ANOVA; $P < 0.05$) in total SOD activity were seen between spermatozoa samples incubated with and without SP at all studied X–XO concentrations. In addition, a gradual increase (ANOVA; $P < 0.05$) in SOD activity was observed with increasing concentrations of X–XO and duration of incubation. Combinations of X–XO + SOD, X–XO + CAT, and X–XO + GTH mitigated spermatozoa SOD activity (Fig. 5).

Glutathione reductase activity showed a trend similar to that of SOD (Fig. 6), and significant
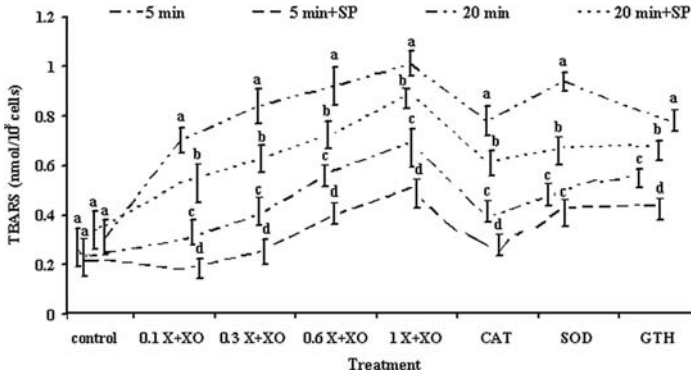
Fig. 3 Effect of antioxidants and seminal plasma on TBARS level in common carp *Cyprinus carpio* spermatozoa exposed to the xanthine–xanthine oxidase (X–XO). *Control* indicates control group where sperm samples were incubated for 5 or 20 min in the IM (200 mM KCl, 30 mM Tris–HCl, pH 8.0) with or without of SP. Data are presented as mean values ± SD. *Different letters* indicate significant differences among samples (ANOVA, $P < 0.05$)
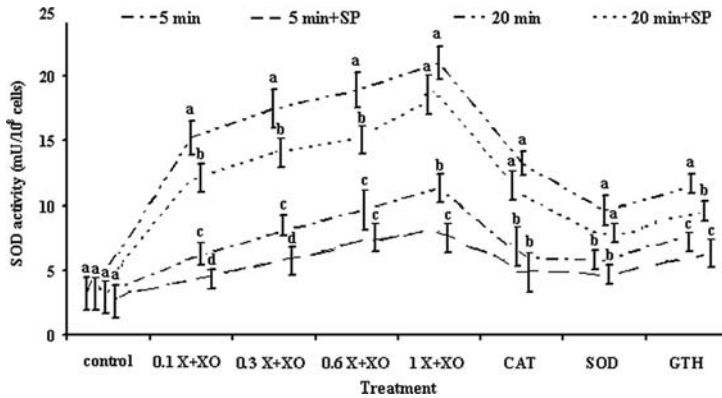
Fig. 4 Effect of antioxidants and seminal plasma on CP level in common carp *Cyprinus carpio* spermatozoa exposed to the xanthine–xanthine oxidase (X–XO) system. *Control* indicates control group where sperm samples were incubated for 5 or 20 min in the IM (200 mM KCl, 30 mM Tris–HCl, pH 8.0) with or without of SP. Data are presented as mean values ± SD. *Different letters* indicate significant differences among samples (ANOVA, $P < 0.05$)
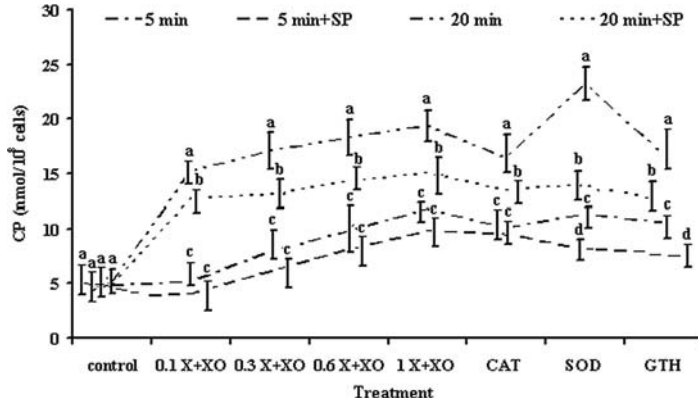
differences (ANOVA; $P < 0.05$) in activity of GR between spermatozoa samples incubated with and without SP were observed at all investigated X–XO concentrations. The highest value of GR activity (26.15 ± 1.98 mU/10^8 cells) was recorded in spermatozoa incubated with 1 mM X–0.1 U/mL XO for 20 min. No significant ($P > 0.05$) differences in GR activity were detected among spermatozoa samples held for 5 min with X–XO + SOD, X–XO + CAT, or X–XO + GTH with or without SP. After 5 min of incubation at 0.1 mM X–0.0125U/mL XO, GR

activity of spermatozoa reached 9.5 mU/10^8 compared with the control where 6.1 mU/10^8 was observed.

The minimum detected GPx activity was 2.3 mU/10^8 in spermatozoa exposed to 0.1 mM X–0.0125 U/mL XO with SP and incubated for 5 min, whereas the maximum (17.51 ± 1.41 mU/10^8 cells) was observed in samples incubated with 1 mM X–0.1 U/mL XO for 20 min without SP. Spermatozoa subjected to the action of 1 mM X–0.1 U/mL XO combined with CAT, SOD, or GTH showed significantly higher activity of GPx

Protection of common carp (Cyprinus carpio L.) spermatozoa
motility under oxidative stress by antioxidants and seminal plasma

Fish Physiol Biochem (2014) 40:1771–1781   1777

Fig. 5 Influence of antioxidants and seminal plasma on SOD activity in common carp *Cyprinus carpio* spermatozoa exposed to the xanthine–xanthine oxidase (X–XO) system. *Control* indicates control group where sperm samples were incubated for 5 or 20 min in the IM (200 mM KCl, 30 mM Tris–HCl, pH 8.0) with or without of SP. Data represent mean values ± SD. *Different letters* indicate statistical significance among samples (ANOVA, $P < 0.05$)
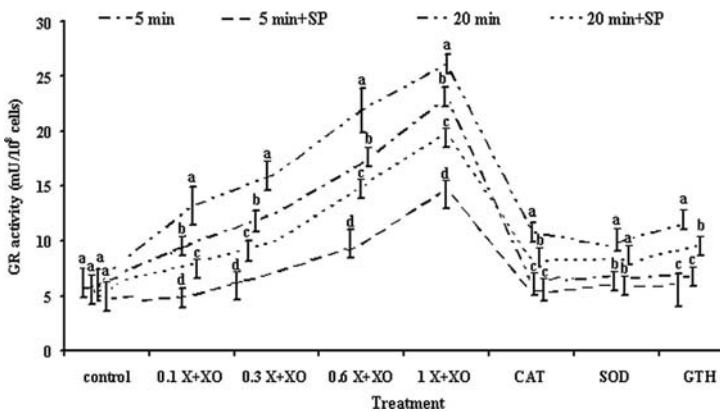
Fig. 6 Influence of antioxidants and seminal plasma on GR activity in common carp *Cyprinus carpio* spermatozoa exposed to the xanthine–xanthine oxidase (X–XO) system. *Control* indicates control group where sperm samples were incubated for 5 or 20 min in the IM (200 mM KCl, 30 mM Tris–HCl, pH 8.0) with or without of SP. Data represent mean values ± SD. *Different letters* indicate statistical significance among samples (ANOVA, $P < 0.05$)

compared with samples exposed to the same combinations plus SP (Fig. 7).

Discussion

As a first step toward understanding how SP and antioxidants may protect sperm exposed to the X–XO

system, oxidative stress indices, antioxidant activity, and spermatozoa motility and velocity of common carp sperm were assessed.

Over the past two decades, considerable knowledge has been accumulated concerning the mechanism of action of X–XO. It was demonstrated that the X–XO system generates ROS that are involved in spermatozoa damage (Griveau et al. 1995), resulting in
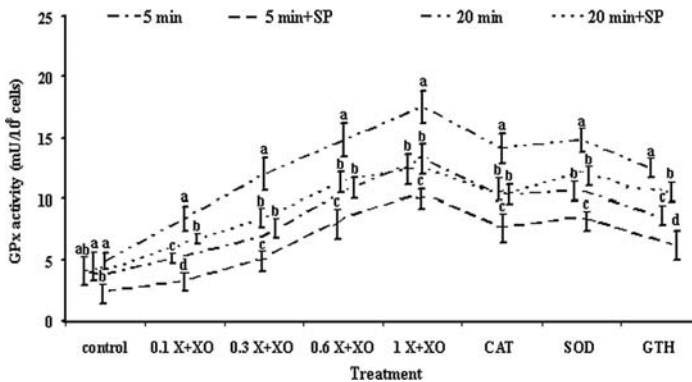
Fig. 7 Influence of antioxidants and seminal plasma on GPx activity in common carp *Cyprinus carpio* spermatozoa exposed to the xanthine–xanthine oxidase (X–XO) system. *Control* indicates control group where sperm samples were incubated for 5 or 20 min in the IM (200 mM KCl, 30 mM Tris–HCl, pH 8.0) with or without of SP. Data represent mean values ± SD. *Different letters* indicate statistical significance among samples (ANOVA, $P < 0.05$)

decreased motility, presumably associated with a rapid loss of intracellular ATP, axoneme injury, and mid-piece morphological defects (Sikka 1996; Bansal and Bilaspuri 2007). In the present study, we found common carp spermatozoa to be activated immediately after transfer to swimming medium, and 95–100 % spermatozoa became motile, a result that agrees with the previous observations (Cosson et al. 2000; Shaliutina et al. 2012). Spermatozoa motility decreased after exposure to varying concentrations of X–XO. Sperm samples exposed to the highest levels of X–XO and incubated for 20 min demonstrated a significant loss of motility and velocity. The decrease in spermatozoa motility parameters after X–XO treatment has several possible explanations. de Lamirande and Gagnon (1992) suggested that spermatozoa motility may be the most sensitive indicator of oxidative stress, because high levels of ROS inhibit one or more enzymes of oxidative phosphorylation and/or glycolysis, limiting the generation of ATP. In the present work, common carp spermatozoa exposed to the X–XO demonstrated increased levels of LPO and protein carbonylation, indicating that oxidative stress was induced by the X–XO system. The percentage of motile spermatozoa and spermatozoa velocity was slightly increased after addition of the antioxidants CAT, SOD, or GTH compared with the motility and velocity of sperm exposed to X–XO alone. These results are in agreement with findings

reported in zebrafish *Danio rerio* by Hagedorn et al. (2012), which showed that CAT improved viability and motility of spermatozoa exposed to the products of the X–XO system. Spermatozoa have limited antioxidant capacity to defend against ROS attack and are highly dependent on their intrinsic intracellular antioxidants and the antioxidant protection of the SP (Koziorowska-Gilun et al. 2011). The present study showed that the addition of SP to spermatozoa samples exposed to the X–XO system and to combinations of X–XO + CAT, X–XO + SOD, or X–XO + GTH significantly increased spermatozoa movement. This has led us to hypothesize that SP, even in small amounts, possesses the capacity to protect sperm from the harmful consequences of oxidative attack. SP also seems to have stronger capacity to maintain a relatively neutral and protective environment for sperm function compared with intracellular components.

Lipid peroxidation occurs when polyunsaturated lipids undergo oxidative damage (Aitken et al. 1995). The high polyunsaturated fatty acid content of the spermatozoon membrane makes it susceptible to such damage (Trenzado et al. 2006). Lipid peroxidation in spermatozoa leads to loss of membrane integrity, causing increased cell permeability, enzyme inactivation, and resistance to osmotic shock, resulting in decreased fertilization potential (Shiva et al. 2011). In addition, protein oxidization may be detrimental to

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sperm function, with plasma membrane structural proteins as well as proteins having enzymatic activity being affected (Domínguez-Rebolledo et al. 2010). Lipid peroxidation is particularly dangerous for aquatic animals, since they normally contain greater amounts of highly unsaturated fatty acids than are found in other species. Lipid peroxidation has been reported to be a major contributor to the loss of cell function under oxidative stress (Storey 1996) and is generally assessed in fish by level of TBARS (Oakes and Van der Kraak 2003). The maximum level of TBARS in the present study was in spermatozoa exposed to the X–XO system at the concentration 1 mM–0.1 U/mL and incubated for 20 min. This is not surprising, since a major mechanism of X–XO action is LPO and membrane disruption (Choi et al. 1996). In an effort to protect spermatozoa from the damaging effects of the X–XO system, SP, the enzymatic antioxidants such as CAT and SOD, and non-enzymatic antioxidant GTH, was added. CAT together with SP in the incubation medium significantly reduced the cytotoxic effect of the X–XO system and decreased the TBARS level, providing evidence that CAT is a powerful antioxidant that can intercept lipid peroxyl radicals and break the chain of LPO (Hagedorn et al. 2012), as was clearly observed when this antioxidant was added to spermatozoa in the presence of ROS. CAT in combination with SP seems to play a more significant role in protection against LPO than does SOD or GTH (Link and Riley 1988).

Increase in CP level also indicates oxidative stress in the sperm cell and is the most frequently used marker of protein oxidation (Li et al. 2010). There is evidence that oxidative modification leads to proteolytic degradation, which may affect the structure, function, and integrity of proteins (Zhang et al. 2008). However, in our previous work (Shaliutina et al. 2013a, b), we concluded that the low level of oxidative stress indices in SP seems to play no role in cellular metabolism (e.g., oxidative phosphorylation) of sperm, which could lead to subsequent decline of motility variables. In the present study, the tested concentrations of X–XO (1 mM–0.1 U/mL), in combination with SOD, were associated with a significant increase in CP level after 20 min exposure, whereas a noticeable decrease in CP level was observed in spermatozoa samples incubated in the presence of SP. Our results also suggested that CP was more sensitive than LPO as an indicator of oxidative stress in

common carp spermatozoa, but further investigation is needed to explore possible reasons.

Fish spermatozoa are equipped with a system of antioxidant defense against ROS attack. Antioxidants represent the primary defense against oxidative stress induced by free radicals and are critical to maintaining sperm viability (Ciereszko et al. 2000). However, the precise mechanism of action of antioxidant systems in fish sperm remains unknown (Lahnsteiner et al. 2010). In the current work, antioxidant activity was evaluated as total SOD, GR, and GPx activity. SOD is considered the first line of defense against the effects of oxyradicals in the cell through catalyzing the dismutation of superoxide radicals to hydrogen peroxide and molecular oxygen (Li et al. 2010). Combinations of X–XO + SOD, X–XO + CAT, or X–XO + GTH were found to mitigate spermatozoa SOD activity. Addition of SP provided better results. It is likely that the abatement of SOD activity in common carp spermatozoa is compensated for by the addition of other enzymatic antioxidants and by the antioxidant properties of SP proteins.

The enzyme system comprising GR provides defense against LPO in mammalian sperm, and defects in the activity of this enzyme can lead to a loss of cell function (Cheema et al. 2009). Our results showed that the addition of CAT, SOD, or GTH had a positive effect on sperm GR activity. Lahnsteiner et al. (2011) demonstrated reduction in the damaging effects of ROS by the addition of antioxidant compounds to the freezing media prior to cryopreservation of fish sperm; however, the influence 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).

Glutathione peroxidase, a ROS scavenger, plays a role in protecting spermatozoon membranes from the deleterious effects of LPO (Alvarez and Storey 1989). Additionally, GPx can inhibit LPO of the cell membrane (Sharma and Agarwal 1996). Our previous study (Shaliutina et al. 2013a, b) presented little differences in GPx level between SP and spermatozoa in common carp that likely is an adaptive response to toxicant stress and serves to neutralize the impact of increased ROS generation. In the present study, a significant enhancement of GPx activity was observed with increasing concentration of X–XO. We hypothesize that the increase in GPx activity was an adaptive response to toxic stress and served to neutralize the

impact of increased ROS generation. The presence of the investigated antioxidants in the incubation system did not significantly affect GPx activity, while the addition of SP reduced activity of GPx in all tested samples. Our results are in agreement with those observed in humans (Aitken et al. 1993).

Conclusions

The study demonstrated that common carp spermatozoa are highly sensitive to the effects of the X–XO system. The X–XO system induces oxidative stress in spermatozoa leading to reduced motility and velocity. However, the use of CAT, SOD, or GTH in combination with SP showed that SP plays an important role in the reduction of ROS induced by X–XO. Further studies should determine whether the protection afforded by SP is correlated with its antioxidant properties, and this work is in progress in our laboratory.

References

Aitken RJ, Buckingham D, Harkiss D (1993) Use of a xanthine oxidase free radical generating system to investigate the cytotoxic effects of reactive oxygen species on human spermatozoa. J Reprod Fertil 97:441–450

Aitken RJ, Paterson M, Fisher H, Buckingham DW, van Duin M (1995) Redox regulation of tyrosine phosphorylation in human spermatozoa and its role in the control of human sperm function. J Cell Sci 108:2017–2025

Aitken RJ, Gordon E, Harkiss D, Twigg JP, Milne P, Jennings Z, Irvine DS (1998) Relative impact of oxidative stress on the functional competence and genomic integrity of human spermatozoa. Biol Reprod 59:1037–1046

Alvarez JG, Storey BT (1989) Role of glutathione peroxidase in protecting mammalian spermatozoa from loss of motility caused by spontaneous lipid peroxidation. Gamete Res 23:77–90

Bansal AK, Bilaspuri GS (2007) Effect of ferrous ascorbate on in vitro capacitation and acrosome reaction in cattle bull spermatozoa. Anim Sci Rep 1:69–77

Bansal AK and Bilaspuri GS (2010) Impacts of oxidative stress and antioxidants on semen functions. Vet Med Int 37:61–68

Baumber J, Ball BA, Gravance CG, Medina V, Davies-Morel MC (2000) The effect of reactive oxygen species on equine sperm motility, viability, acrosomal integrity, mitochondrial membrane potential, and membrane lipid peroxidation. J Androl 21:895–902

Cabrita E, Diogo P, Martínez-Páramo S, Sarasquete C, Dinis MT, Dinis MT (2011) The influence of certain aminoacids and vitamins on post-thaw fish sperm motility, viability and DNA fragmentation. Anim Reprod Sci 125:189–195

Caille N, Rodina M, Kocour M, Gela D, Flajshans M, Linhart O (2006) Quality, motility and fertility of tench (*Tinca tinca*) sperm in relation to LHRH analogue and carp pituitary treatments. Aquacult Int 4:75–87

Carlberg I, Mannervik B (1975) Purification and characterization of flavoenzyme glutathione reductase from rat liver. Biol Chem 250:5475–5480

Cheema RS, Bansal AK, Bilaspuri GS (2009) Manganese provides antioxidant protection for sperm cryopreservation that may offer new consideration for clinical fertility. Oxidative Med Cell Longev 2:152–159

Choi GJ, Lee HJ, Cho KY (1996) Lipid peroxidation and membrane disruption by vinclozolin in dicarboximide-susceptible and -resistant isolates of *Botrytis cinerea*. Pestic Biochem Physiol 55:29–39

Ciereszko A, Dabrowski K (1995) Sperm quality and ascorbic acid concentration in rainbow trout semen are affected by dietary vitamin C: an across-season study. Biol Reprod 52:982–988

Ciereszko A, Dabrowski K, Kuchrczyk MJ, Dobosz S, Goryczko K, Glogowski J (1999) The presence of uric acid, an antioxidative substance, in fish seminal plasma. Fish Physiol Biochem 21:313–315

Ciereszko A, Glogowski J, Dabrowski K (2000) Biochemical characteristics of seminal plasma and spermatozoa of freshwater fishes. In: Tiersch TR, Mazik PM (eds) Cryopreservation in aquatic species. World Aquaculture Society, Baton Rouge, pp 20–48

Cosson J, Linhart O, Mims S, Shelton W, Rodina M (2000) Analysis of motility parameters from paddlefish (*Polyodon spathula*) and shovelnose sturgeon (*Scaphirhynchus platorynchus*) spermatozoa. J Fish Biol 56:1348–1367

de Lamirande E, Gagnon C (1992) Reactive oxygen species and human spermatozoa. I. Effect on the motility of intact spermatozoa and on sperm axonemes. J Androl 13:368–378

de Lamirande E, Gagnon C (1993) A positive role for the superoxide anion in triggering hyperactivation and capacitation of human spermatozoa. Int J Androl 16:21–25

de Lamirande E, Gagnon C (1995) Capacitation-associated production of superoxide anion by human spermatozoa. Free Radic Biol Med 18:487–496

Domínguez-Rebolledo ÁE, Fernández-Santos MR, Bisbal A, Ros-Santaella JL, Ramón M, Carmona M (2010) Improving the effect of incubation and oxidative stress on thawed spermatozoa from red deer by using different antioxidant treatments. Reprod Fertil Dev 22:856–870

Gazo I, Linhartova P, Shaliutina A, Hulak M (2013) Influence of environmentally relevant concentrations of vinclozolin on

*Protection of common carp (Cyprinus carpio L.) spermatozoa
motility under oxidative stress by antioxidants and seminal plasma*

Fish Physiol Biochem (2014) 40:1771–1781   1781

sperm quality, DNA integrity, and antioxidant responses in sterlet *Acipenser ruthenus* spermatozoa. Chem Biol Interact 203:377–385

Griveau JF, Renard P, Le Lannou D (1995) Superoxide anion production by human spermatozoa as a part of the ionophore-induced acrosome reaction process. Int J Androl 18:67–74

Hagedorn M, McCarthy M, Carter VL, Meyers SA (2012) Oxidative stress in zebrafish (*Danio rerio*) sperm. PLoS One 7(6):e39397

Koppers AJ, Garg ML, Aitken RJ (2010) Stimulation of mitochondrial reactive oxygen species production by unesterified, unsaturated fatty acids in defective human spermatozoa. Free Radic Biol Med 48:112–119

Koziorowska-Gilun M, Koziorowski M, Fraser L, Strzeżek J (2011) Antioxidant defence system of boar caudaepididymidal spermatozoa and reproductive tract fluids. Reprod Domest Anim 46(3):527–533

Lahnsteiner F, Mansour N, Plaetzer K (2010) Antioxidant systems of brown trout (*Salmo trutta f. fario*) semen. Anim Reprod Sci 119:314–321

Lahnsteiner F, Mansour N, Kunz FA (2011) The effect of antioxidants on the quality of cryopreserved semen in two salmonid fish, the brook trout (*Salvelinus fontinalis*) and the rainbow trout (*Oncorhynchus mykiss*). Theriogenology 76:882–890

Lawrence RA, Burk RF (1976) Glutathione peroxidase activity in selenium deficient rat liver. Biochem Biophys Res Commun 71:952–958

Lenz AG, Costabel U, Shaltiel S, Levine RL (1989) Determination of carbonyl groups in oxidatively modified proteins by reduction with tritiated sodium borohydride. Anal Biochem 177:419–425

Li P, Li ZH, Dzyuba B, Hulak M, Rodina M, Linhart O (2010) Evaluating the impacts of osmotic and oxidative stress on common carp (*Cyprinus carpio,* L.) sperm caused by cryopreservation techniques. Biol Reprod 83:852–858

Link EM, Riley PA (1988) Role of hydrogen peroxide in the cytotoxicity of the xanthine/xanthine oxidase system. Biochem J 249:391–399

Liu L, Dabrowski K, Ciereszko A (1995) Protective effect of seminal plasma proteins on the degradation of ascorbic acid. Mol Cell Biochem 148:59–66

Lushchak VI, Bagnyukova TV, Lushchak OV, Storey JM, Storey KB (2005) Hypoxia and recovery perturb free radical processes and antioxidant potential in common carp (*Cyprinus carpio*) tissues. Int J Biochem Cell Biol 37:1319–1330

Marklund S, Marklund G (1974) Involvement of superoxide anion radical in autoxidation of pyrogallol and a convenient assay for superoxide dismutase. Eur J Biochem 47:469–474

Martínez-Páramo S, Diogo P, Dinis MT, Herráez MP, Sarasquete C, Cabrita E (2012) Incorporation of ascorbic acid and α-tocopherol to the extender media to enhance antioxidant system of cryopreserved sea bass sperm. Theriogenology 77:1129–1136

Metwally MAA, Fouad IM (2009) Effects of L-ascorbic acid on sperm viability in male grass carp (*Ctenopharyngodon idellus*). Glob Vet 3(2):132–136

Oakes KD, Van der Kraak GJ (2003) Utility of the TBARS assay in detecting oxidative stress in white sucker (*Catostomus commersoni*) populations exposed to pulp mill effluent. Aquat Toxicol 63:447–463

Olson JS, Ballou DP, Palmer G, Massey V (1974) The reaction of xanthine oxidase with molecular oxygen. J Biol Chem 249:4350

Rodina M, Gela D, Kocour M, Alavi SMH, Hulak M, Linhart O (2007) Cryopreservation of tench, *Tinca tinca*, sperm: sperm motility and hatching success of embryos. Theriogenology 67:931–940

Saleh RA, Agarwal A (2002) Oxidative stress and male infertility: from research bench to clinical practice. J Androl 23:737–752

Shaliutina A, Hulak M, Dzuyba B, Linhart O (2012) Spermatozoa motility and variation in the seminal plasma proteome of Eurasian perch (*Perca fluviatilis*) during the reproductive season. Mol Reprod Dev 79(12):879–887

Shaliutina A, Hulak M, Gazo I, Linhartova P, Linhart O (2013a) Effect of short-term storage on quality parameters, DNA integrity, and oxidative stress in Russian (*Acipenser gueldenstaedtii*) and Siberian (*Acipenser baerii*) sturgeon sperm. Anim Reprod Sci 139(1–4):127–135

Shaliutina A, Gazo I, Cosson J, Linhart O (2013b) Comparison of oxidant and antioxidant status of seminal plasma and spermatozoa of several fish species. Czech J Anim Sci 58(7):313–320

Sharma RK, Agarwal A (1996) Role of reactive oxygen species in male infertility. Urology 48:835–850

Sharma RK, Pasqualotto FF, Nelson DR, Thomas AJ, Agarwal A (1999) The reactive oxygen species—total antioxidant capacity score is a new measure of oxidative stress to predict male infertility. Hum Reprod 14:2801–2807

Shiva M, Gautam AK, Verma Y, Shivgotra V, Doshi H, Kumar S (2011) Association between sperm quality, oxidative stress, and seminal antioxidant activity. Clin Biochem 44:319–324

Sikka SC (1996) Oxidative stress and role of antioxidants in normal and abnormal sperm function. Front Biosci 1:78–86

Sikka SC (2001) Relative impact of oxidative stress on male reproductive function. Curr Med Chem 8:851–862

Storey KB (1996) Oxidative stress: animal adaptations in nature. Braz J Med Biol Res 29:715–1733

Trenzado C, Hidalgo MC, García-Gallego M, Morales AE, Furné M, Domezain A, Domezain J, Sanz A (2006) Antioxidant enzymes and lipid peroxidation in sturgeon *Acipenser naccarii* and trout *Oncorhynchus mykiss*. A comparative study. Aquaculture 254:758–767

Zhang XZ, Xie P, Wang WM, Li DP, Shi ZC (2008) Plasma biochemical responses of the omnivorous crucian carp (*Carassius auratus*) to crude cyanobacterial extracts. Fish Physiol Biochem 34:323–329

THE EFFECT OF REACTIVE OXYGEN SPECIES ON MOTILITY PARAMETERS, DNA INTEGRITY, TYROSINE PHOSPHORYLATION AND PHOSPHATASE ACTIVITY OF COMMON CARP (*CYPRINUS CARPIO* L.) SPERMATOZOA

The Effect of Reactive Oxygen Species on Motility Parameters, DNA Integrity, Tyrosine Phosphorylation and Phosphatase Activity of Common Carp (*Cyprinus carpio* L.) Spermatozoa

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SUMMARY

The effect of reactive oxygen species production on the motility parameters, DNA integrity, acid phosphatase activity, and protein tyrosine phosphorylation in spermatozoa of the common carp (*Cyprinus carpio*) was investigated. Spermatozoa were exposed to different concentrations of xanthine and xanthine oxidase (X-XO) either in the presence or absence of antioxidants for 15 and 60 min. A dose- and time-dependent reduction in spermatozoa motility and velocity was observed. Comet assays showed a dramatic increase in DNA fragmentation after 15 min. Changes in tyrosine phosphorylation of spermatozoa proteins were observed by Western blotting with anti-phosphotyrosine antibodies, and proteins of interest were identified by mass spectrometry. After a 60 min exposure to X-XO, O-linked N-acetylglucosamine transferase, isoform 4 was phosphorylated and septin-8-A was dephosphorylated. Acid phosphatase activity also decreased in a dose-dependent manner after a 60 min exposure to oxidative stress. The results demonstrate that oxidative stress impaired functional variables (sperm motility, velocity, DNA integrity) of carp spermatozoa, and altered intracellular signalling pathways through changes in tyrosine phosphorylation and acid phosphatase activity.

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INTRODUCTION

Sperm cells of externally fertilizing species, including most fish, are particularly vulnerable to oxidative stress when released into the aquatic environment because of

Abbreviations: ROS, reactive oxygen species; SOD, superoxide dismutase; X-XO, xanthine-xanthine oxidase

the insufficient protection provided by their membranes. In sperm of freshwater teleosts, the hypotonic shock required for motility also enhances production of reactive oxygen species (ROS) as well as a variety of other deleterious compounds such as quinones (Morisawa and Suzuki, 1980; Zhou et al., 2006). This situation contrasts with internal fertilization, such as used by mammals, which offers better protection of spermatozoa against the overwhelming aggression of ROS during their transit in the genital tracts. In fish, spermatozoa cryopreservation (Li et al., 2013) and short-term storage (Shaliutina et al., 2013) may also increase oxidative stress, leading to sub-lethal or lethal spermatozoa damage.

ROS are short-lived, highly reactive chemical species (atoms or small molecules) that include oxygen ions and peroxides. The high reactivity of ROS is a consequence of unpaired valence-shell electrons, which can generate significant damage to cellular structures. ROS may be produced endogenously by intracellular oxidase and peroxidase enzymes, such as xanthine oxidase and nitric oxide synthase, or are by-products of the activity of other enzymes, such as cytochrome p450. Indeed, the main source of ROS in most cells is electron leakage from the inner mitochondrial membrane during oxidative phosphorylation and ATP generation (Ford, 2004; Ray et al., 2012).

Excessive levels of ROS from exogenous or endogenous sources have been shown to be harmful to spermatozoa, as they lead to lipid peroxidation of the plasma membrane. In human spermatozoa, this damage compromises cellular function, decreases DNA integrity, and alters protein conformation (Agarwal et al., 2003; Agarwal and Said, 2005). Studies of mammalian spermatozoa have shown that low concentrations of ROS trigger sperm cell-signalling events (cAMP production) and regulate physiological functions, such as capacitation and the acrosome reaction (OFlaherty et al., 2006). Spermatozoa of the common carp, however, do not require cAMP for motility and lack an acrosome (Krasznai et al., 2000). Thus the influence of ROS on carp spermatozoa may be different from the one utilized by mammals or other species possessing a sperm acrosome.

Fish spermatozoa are generally characterized by a short motility period—less than 60 sec in some species—so the level of sperm motility achieved becomes a key contribution to the successful delivery of paternal DNA to an egg. Oxidative stress, however, may impair sperm motility as well as DNA integrity (Zhou et al., 2006). The importance of these parameters and their sensitivity to the presence of free radicals makes them good gauges of ROS level, although the ROS mechanism of action and effect on intracellular signalling pathways in fish spermatozoa remain to be elucidated.

Protein tyrosine phosphorylation is one of the most extensively studied post-translational modifications in spermatozoa (OFlaherty et al., 2006). Tyrosine phosphorylation of sperm proteins plays a crucial role in the regulation of flagellar motility in spermatozoa of various species (Visconti et al., 1995; Inaba, 2003). The phosphorylation status of proteins on serine/threonine (Ser/Thr) and tyrosine (Tyr) residues is controlled by the reciprocal activity of protein kinases and protein phosphatases, which are influenced by cellular redox status (Aitken et al., 1995; Ray et al., 2012).

The xanthine-xanthine oxidase (X-XO) system generates a mixture of superoxide anion (O_2^-) and hydrogen peroxide (H_2O_2) (Aitken et al., 1993). The scavenging enzymes superoxide dismutase (SOD) and catalase selectively remove super oxide anion (O_2^-) and hydrogen peroxide (H_2O_2), respectively, from the cytosol. In combination, the X-XO system and antioxidant enzymes allows for the investigation of superoxide anion and hydrogen peroxide effects on fish spermatozoa. In the present study, the effects of ROS production on motility and DNA integrity of spermatozoa from the freshwater fish species *Cyprinus carpio* L. (the common carp) were tested. To explore the role of free radicals in spermatozoa intracellular signalling, we examined the influence of ROS on tyrosine phosphorylation and acid phosphatase activity.
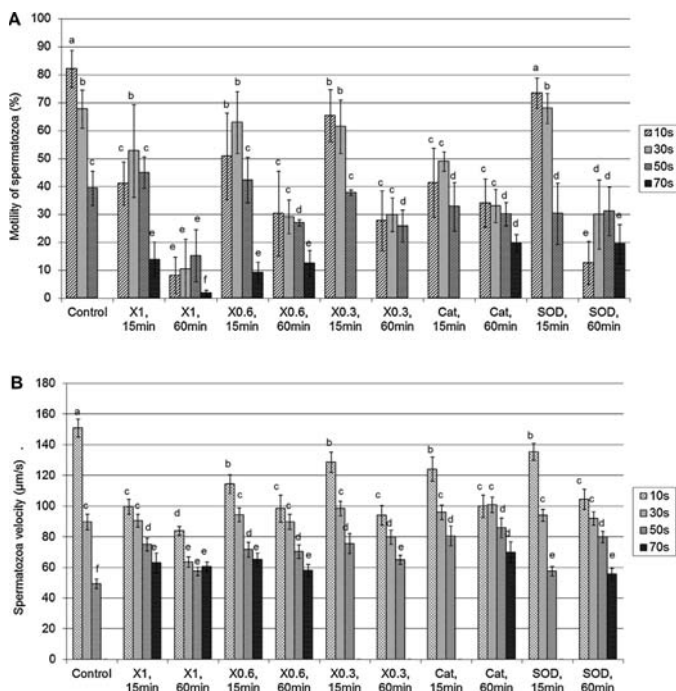
RESULTS

The Effect of ROS Production on Spermatozoa Motility and Velocity

The effect of ROS production on the percentage of motile spermatozoa and their curvilinear velocity was assessed at 10, 30, 50, and 70 sec post-activation (Fig. 1). In the presence of the X-XO system, motility rate and velocity of spermatozoa decreased in a time- and dose-dependent manner compared to the control. More than 80% of the spermatozoa were motile at 10 sec post-activation (mean velocity, $150 \pm 5.8\,\mu m/sec$) in the control group, whereas only 41% of the population was motile (mean velocity, $99 \pm 4.9\,\mu m/sec$) after 15 min incubation with 1 mM X-0.1 U/ml XO, and fewer than 10% of spermatozoa were motile (mean velocity, $83 \pm 3\,\mu m/sec$) after 60 min incubation.

Oxidative stress also induced a delay in the activation of motility. In the control group, the highest rate of motile spermatozoa was observed at 10 sec post-activation and endured until 50 sec post-activation. In contrast, spermatozoa exposed to 1 mM X-0.1 U/ml XO and 0.6 mM X-0.05 U/ml XO achieved maximum motility at 30 sec and remained high up to 70 sec post-activation.

Pre-incubation with the antioxidant enzymes catalase and SOD partially prevented the reduced spermatozoa motility induced by X-XO. Spermatozoa pre-incubated with 200 U/ml catalase showed 34% motile spermatozoa (mean velocity, $99.8 \pm 7.3\,\mu m/sec$) after 60 min of incubation in 1 mM X-0.1 mM XO, which is significantly higher than observed in spermatozoa exposed to X-XO only. SOD was slightly less effective in protecting spermatozoa against X-XO-induced damage: following a 60 min incubation with the ROS generating system, a

The effect of reactive oxygen species on motility parameters, DNA integrity, tyrosine phosphorylation and phosphatase activity of common carp (Cyprinus carpio L.) spermatozoa

Molecular Reproduction & Development

GAZO *ET AL.*

Figure 1. The effect of ROS levels on spermatozoa motility (A) and velocity (B) in the common carp. Data are presented as means ± standard deviation. Superscripts indicate significant differences among samples (ANOVA, $P < 0.05$).

delay in motility activation was observed in spermatozoa pre-incubated with 200 U/ml SOD. The highest rate of spermatozoa motility was 30%, observed at 50 sec post-activation.

Spermatozoa DNA Integrity Under Oxidative Stress

Comet-assay analysis indicated a dramatic increase in DNA fragmentation in spermatozoa nuclei after a 15-min exposure to 1 mM X-0.1 U/ml XO (Fig. 2). After a 60-min exposure, DNA fragmentation reached 25% whereas 8.6% was observed in the control. Similar to spermatozoa motility and velocity, DNA integrity was declined by ROS in a dose- and time-dependent manner.

Pre-incubation with antioxidant enzymes resulted in significantly lower DNA fragmentation, with only 17−19% of nuclear DNA showing impairment after a 60 min incubation with X-XO. No difference was observed between samples with SOD and catalase, suggesting that the

protective potential of SOD and catalase against DNA fragmentation is the same.

**The Effect of X-XO on Protein Tyrosine Phosphorylation**

Loss of spermatozoa motility and DNA fragmentation suggest that incubation of spermatozoa for 60 min with X-XO leads to prominent oxidative damage. In order to identify the effects of this oxidative damage, protein phosphorylation was evaluated (Fig. 3). Incubation with phospho-tyrosine antibodies revealed changes in the phosphorylation status of two proteins: a 110-kDa protein was phosphorylated while a 48-kDa protein was dephosphorylated in the presence of X-XO.

Proteins extracted from control spermatozoa and spermatozoa exposed to 1 mM X-0.1 U/ml XO were separated by 2-dimensional gel electrophoresis, blotted, and probed with anti-phosphotyrosine antibodies to identify which proteins were involved in the response to oxidative stress
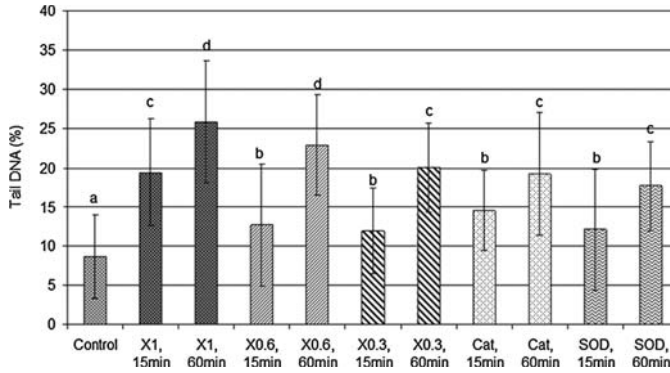
Figure 2. Influence of the X-XO system on DNA fragmentation in carp spermatozoa, as measured by the comet assay. Data represent mean percentage of DNA in the tail ± standard deviation. Different letters indicate statistical significance among samples (ANOVA, $P < 0.05$).

(Fig. 4). These two-dimensional gel immunoblots revealed that the 110-kDa band was not a single protein but a cluster of proteins with the same molecular weight but different isoelectric points (pI) (6.5–6.75) (Figs. 4 and 5).
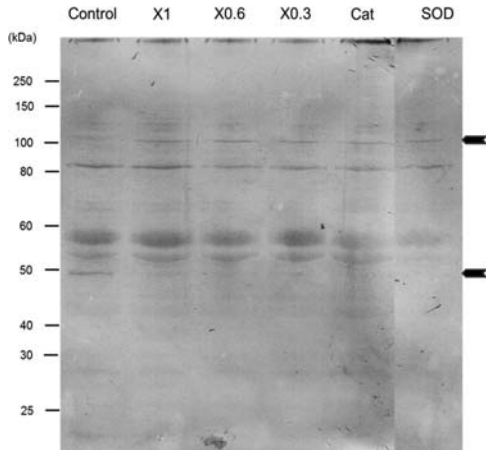
Figure 3. Western-blot analysis with anti-phosphotyrosine antibodies of carp spermatozoa proteins separated by SDS-PAGE. Control indicates the untreated group; X1–X0.3 indicates spermatozoa exposed to different concentrations of X-XO in immobilizing medium for 1 hr; SOD and Cat indicates spermatozoa pre-incubated with 200 U/ml of SOD or catalase, respectively, and then subsequently exposed to 1 mM xanthine plus 0.1 U/ml xanthine oxidase for 1 hr. Spermatozoa proteins were subjected to Western-blot analysis (25 μg per lane) with anti-phosphotyrosine antibodies. The numbers on the left indicate the molecular-weight standard. Positions of proteins of interest that show differential phosphorylation are indicated on the right.

The 48-kDa band was a single protein with pI 6.5 (Figs. 4 and 5).

Among the proteins observed by two-dimensional gel electrophoresis and Western blotting, two spots were identified by mass spectrometry (Table 1). The most neutral 110-kDa protein (Spot 3) was identified as the O-linked N-acetylglucosamine transferase, isoform 4, while the 48-kDa protein (Spot 4) was identified as septin-8-A. Sequences of identified proteins were analyzed with NetPhos 2.0 (http://www.cbs.dtu.dk/services/Net-Phos/), which predicts potential phosphorylation sites in proteins. Fifteen putative phosphorylated tyrosine residues were found in O-linked N-acetylglucosamine transferase, isoform 4, among which 11 have a high score (>0.8); three phosphorylation sites were predicted for septin-8-A, all with scores >0.7.
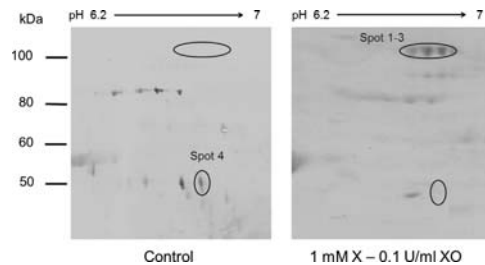
Figure 4. Western-blot analysis with anti-phosphotyrosine antibodies of carp spermatozoa proteins separated using two-dimensional gel electrophoresis. Labelled spots represent proteins that were phosphorylated or dephosphorylated at tyrosine residues.

51

*The effect of reactive oxygen species on motility parameters, DNA integrity, tyrosine phos-
phorylation and phosphatase activity of common carp (Cyprinus carpio L.) spermatozoa*

Molecular Reproduction & Development   Gazo ET AL.

TABLE 1. Protein Identification by MALDI-TOF-MS/MS

Spot	Protein name (organisms)	Accession no.	Mass (kDa)	pI	MASCOT score	Sequence coverage (%)	Number of peptides (ion score $\geq$ 30)
			Calculated				
S3	O-linked N-acetylglucosamine transferase isoform 4 [*Danio rerio*]	gi|66347879|ref| NP_001018117.1|	116,599	6.14	114	13	2
S4	Septin-8-A [Danio rerio]	gi|223635831|sp|Q642H3.2|	49,253	5.90	231	42	2

Acid Phosphatase Activity in Spermatozoa Exposed to Oxidative Stress

To understand the underlying changes in protein tyrosine phosphorylation and dephosphorylation that were induced by ROS production, the activity of acid phosphatase was measured in spermatozoa exposed to oxidative stress. Acid phosphatase activity decreased in a dose-dependent manner after incubation for 60 min with X-XO (Fig. 6). At the highest X-XO concentration, however, acid phosphatase activity was significantly lower (30 U/L) than in the control (38 U/L). The presence of catalase returned activity to control levels. Pre-incubation with SOD, on the other hand, had a minimal effect, retaining phosphatase activity at levels similar to those observed with 1 mM X-0.1 U/ml XO. In presence of the tyrosine phosphatase inhibitor sodium orthovanadate, acid phosphatase activity was significantly lower than in the control.

Figure 5. Two-dimensional gel electrophoresis maps of carp spermatozoa proteins. The separated proteins were stained with Coomassie Brilliant Blue R-250. Labeled spots correspond to proteins phosphorylated and dephosphorylated under oxidative stress.

DISCUSSION

Numerous studies have described the effect of ROS on mammalian spermatozoa (de Lamirande et al., 1997; Aitken and Baker, 2006), but only a few studies have dealt with the role of ROS in fish spermatozoa (Morita et al., 2011; Hagedorn et al., 2012). This is surprising since the spermatozoa of freshwater fish are particularly vulnerable to oxidative injury, as they are delivered into a non-protective environment at spawning.

In the current study using spermatozoa of the common carp, excess ROS led to decreases in sperm motility, velocity, and DNA integrity in a time- and dose-dependent manner. We previously showed that, in the presence of various xenobiotic compounds, both spermatozoa motility and DNA integrity are inversely correlated with indicators of oxidative stress such as lipid oxidation and protein carbonylation (Gazo et al., 2013; Linhartova et al., 2013), so DNA fragmentation is a reliable metric of free-radical mediated damage induced by oxidative stress (Kodoma et al., 1997). The results presented here indicate the accumulation of oxidative damage in a time- and dose-dependent manner in sperm samples incubated with X-XO. Although catalase and SOD partly protected spermatozoa from oxidative damage and significantly decreased the effects of X-XO, indicators of oxidative stress remained in spermatozoa pre-incubated with the antioxidant enzymes. Moreover, a delay in the activation of sperm motility was observed in sperm samples exposed to the highest X-XO concentrations independent of the presence or absence of these antioxidants.

There are several means by which oxidative stress may affect spermatozoa motility and velocity. High membrane concentrations of phospholipids, sterols, and saturated and polyunsaturated fatty acids increase spermatozoa susceptibility to damage induced by excessive ROS production (Sanocka and Kurpisz, 2004). Lipid peroxidation in biological membranes is associated with impairing membrane function, decreasing fluidity, inactivating membrane-bound receptors and enzymes, and increasing non-specific permeability to ions (Wong-Ekkabut et al., 2007). Increased membrane permeability in particular can disrupt ion gradients, altering the membrane potential necessary for sperm motility activation (Boitano and Omoto, 1991). Products of lipid peroxidation are also biologically active and can cause severe cell dysfunction at the DNA level (Sanocka
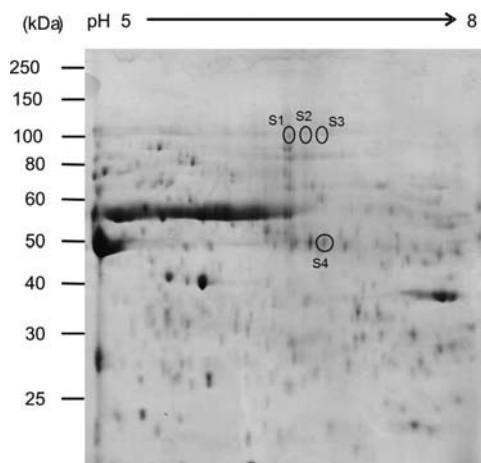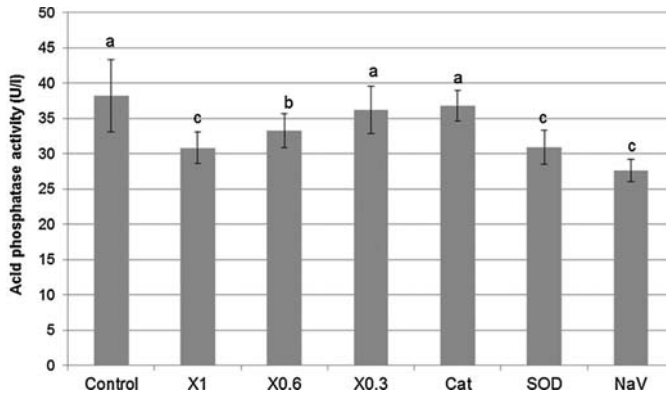
52   Mol. Reprod. Dev. 82:48–57 (2015)

- 63 -

Figure 6. Acid phosphatase activity in common carp spermatozoa incubated with different concentrations of X-XO, antioxidant enzymes (SOD or catalase [Cat]), or sodium orthovanadate (NaV) for 1 hr. Data represent mean values ± standard deviation. Different letters indicate statistical significance among samples (ANOVA, $P < 0.05$).

and Kurpisz, 2004). In mammalian spermatozoa, increased ROS production is additionally associated with decreased mitochondrial membrane potential and ATP content (Chance et al., 1979; Wang et al., 2003). All these events can delay the activation of motility as well as decrease the velocity and the percentage of motile spermatozoa.

Activation of sperm motility is a complex process involving a cascade of intracellular reactions mediated by proteins (Inaba, 2003). As proteins are also vulnerable to oxidative damage, one might expect that intracellular signalling pathways and spermatozoa movement would also be adversely affected (DuTeaux et al., 2004). In the present work, even low doses of free radicals (0.3 mM X-0.025 U/ml XO) were observed to alter the phosphorylation state of O-linked *N*-acetylglucosamine transferase and septin-8-A, suggesting that these proteins are especially sensitive to ROS since they were modified by all tested concentrations of X-XO both in the presence and absence of antioxidant enzymes.

The O-linked *N*-acetylglucosamine transferases (OGTs) are eukaryotic enzymes that add a single O-linked β-N-acetylglucosamine (O-GlcNAc) moiety to target proteins. O-GlcNAc exhibits a complex relationship with phosphate modifications, where they can competitively occupy a single or proximal site, or non-competitively occupy different sites on a substrate (Zeidan and Hart, 2010). Phosphorylation regulates O-GlcNAc-cycling enzymes and, conversely, O-GlcNAcylation controls phosphate-cycling enzymes. Studies using insulin-treated adipocytes have shown that tyrosine phosphorylation is one of the mechanisms controlling the catalytic activity of OGTs (Whelan et al., 2008). O-GlcNAc is also involved in the acrosome reaction in mammalian spermatozoa as well as in cell-cell interaction during gamete fusion (Wu and Sampson, 2014). Furthermore, multiple cell lines have been reported to increase levels of O-GlcNAc and of OGT activity in response to osmotic, ethanolic, and oxidative stress (Zachara et al., 2004). Together, these activities are consistent with a function for OGT in fish spermatozoa and lead to the hypothesis that a rapid, ROS-dependent increase of OGT tyrosine phosphorylation may mediate O-GlcNAcylation of sperm proteins and stress-induced signal transduction pathways.

Septin-8-A is a member of the highly conserved septin family of GTP-binding proteins that co-localize with cellular membranes and the microtubule cytoskeleton (Spiliotis, 2010). The biological functions of septins are associated with their ability to assemble into filamentous structures that compartmentalize cell membranes (diffusion barriers) and form scaffolds for protein-protein interactions (e.g., between protein kinases and their substrates) (Spiliotis and Nelson, 2006). In mammalian spermatozoa, septin subunits are consistently found in the annulus, an electron-dense ring-shape structure between the midpiece and principle piece of the flagellum. Phosphorylation controls septin-ring dynamics by stabilizing interactions between septin complexes; dephosphorylation corresponds to a fluid state of the septin complex, when subunits move rapidly inside the ring, possibly via the cytoplasm (Dobbelaere et al., 2003). We therefore speculate that oxidative stress cause transitions of septin filaments from an immobile, "frozen" state to a dynamic, fluid state—a process that alters its barrier and scaffold functions. The mechanism by which changes in septin phosphorylation affect flagellar motility is still unclear, however. A normal septin cortex is required for proper organization of the core axonemal

53

The effect of reactive oxygen species on motility parameters, DNA integrity, tyrosine phosphorylation and phosphatase activity of common carp (Cyprinus carpio L.) spermatozoa

Molecular Reproduction & Development
GAZO ET AL.

structure; in spermatozoa with a defective cortex, the dynein motor cannot fully utilize ATP because the defective intraflagellar transport affects its activation (Ihara et al., 2005). Thus, if the subcellular organization of sperm is shared between mammals and fish, the septin-8-A dephosphorylation observed in carp spermatozoa could be directly responsible for the delayed activation of motility.

Tyrosine kinases and phosphatases, the two types of enzymes involved in the regulation of tyrosine phosphorylation, are susceptible to redox regulation (de Lamirande et al., 1997). In maturing rat sperm, for example, physiological concentrations of superoxide radicals indirectly stimulate tyrosine kinase by raising the cAMP concentration, whereas hydrogen peroxide directly stimulates tyrosine kinase and inhibits tyrosine phosphatase (Lewis and Aitken, 2001). Phosphatase activity is critically dependent upon the reduced status of the thiol group on a highly conserved cysteine residue (Cys215 in protein tyrosine phosphatase 1B), which can be directly oxidized by hydrogen peroxide (Hecht and Zick, 1992; Aitken, 1997). Phosphatase activity in carp spermatozoa decreases in the presence of heavy metals such as zinc, copper, mercury, and cadmium (Sarosiek et al., 2009), presumably also acting through a ROS-dependent pathway. Notably in our study, phosphatase activity was significantly reduced in samples pre-incubated with SOD but not catalase, which scavenges hydrogen peroxide. Reduction of acid phosphatase activity in the presence of sodium orthovanadate supports the correlation between this parameter and tyrosine phosphatase activity.

We conclude that spermatozoa of the common carp are highly sensitive to free radicals. Exposure to even low concentrations of X-XO for 15 min led to significant decreases in spermatozoa motility and DNA integrity, which are indicators of oxidative stress. Intracellular signalling pathways were altered during oxidative stress through changes in tyrosine phosphorylation and phosphatase activity. While additional studies are needed to investigate the role of ROS in carp spermatozoa, it is clear that in comparison to mammalian spermatozoa, these gametes could represent an alternative model to study the role of ROS in male fertility and reproduction.

MATERIALS AND METHODS

Broodstock Handling and Sperm Collection

All experiments were performed according to the principles of the Ethical Committee for the Protection of Animals in Research of the University of South Bohemia, Research Institute of Fish Culture and Hydrobiology at Vodnany in the Czech Republic. Sperm was obtained from the adult common carp (n = 6; body weight, 3–4 kg) reared in the aquaculture facility of the Research Institute of Fish Culture and Hydrobiology at the University of South Bohemia. Fish were held in 4 m^3 tanks at a water temperature of 22 °C and dissolved oxygen levels of 6–7 mg/L. Spermiation was stimulated by intramuscular injection of carp pituitary powder dissolved in 0.9% (w/v) NaCl solution

at 2 mg kg^{-1} body weight, 24 hr prior to stripping. Sperm samples were obtained by abdominal massage, and collected directly into 10-ml plastic syringes. Care was taken to avoid contamination with mucus, feces, or water. Samples were stored on ice (0−4 °C) in closed assay tubes prior to analysis. Spermatozoa concentration of each of the six males was estimated microscopically (Olympus BX 41) at 20× using a Burker cell hemocytometer. Average sperm concentration was 55.1 ± 2.2 × 10^9/ml.

Sample Preparation

Separate sperm samples from each of six experimental males were centrifuged at 300g for 30 min at 4 °C to remove seminal plasma. The resulting pellets were diluted in an immobilizing medium (IM) (200 mM KCl, 30 mM Tris-HCl [pH 8.0]) (Perchec et al., 1995) to a final concentration of 5 × 10^8 cells/ml. ROS were generated using the X-XO system. The sperm aliquots were incubated at 4°C for 15 and 60 min with the following treatments: (i) control, IM only; (ii) spermatozoa plus 1 mM X-0.1 U/ml XO in IM; (iii) spermatozoa plus 0.6 mM X-0.05 U/ml XO in IM; or (iv) spermatozoa plus 0.3 mM X-0.025 U/ml XO in IM. To test the effect of superoxide anions and hydrogen peroxide, samples were pre-incubated for 5 min with either 200 U/ml catalase or superoxide dismutase, then subsequently exposed to 1 mM X-0.1 U/ml XO in IM. The incubation period for each sample was 15 and 60 min at 4°C.

Spermatozoa Motility and Velocity Analysis

Spermatozoa velocity (μm/sec) and percentage of motile spermatozoa were determined after triggering sperm motility under dark-field microscopy (Olympus BX 50, Japan). For triggering motility, sperm was diluted in activation medium (AM) (5 mM KCl, 45 mM NaCl, 30 mM Tris [pH 8.2]) at a final dilution of 1:5000. To prevent spermatozoa from sticking to the microscope slide, 0.25% (w/v) pluronic acid (Sigma−Aldrich, Saint Louis, USA) was included in AM. Spermatozoa motility was recorded with a CCD video camera (SONY SSCDC50AP, Japan) mounted on a microscope, using 20× magnification objective lens and illumination by stroboscopic flash (ExposureScope^{TM}). The positions of the heads of spermatozoa were measured from five successive frames and analyzed with a micro-image analyzer (Olympus Micro Image 4.0.1. for Windows). Velocity and motility were assessed at 10, 30, 50, and 70 sec post-activation, as described by Rodina et al. (2007). Measurement of spermatozoa motility for each sample was performed in triplicate.

Comet Assay

DNA integrity was assessed using single-cell gel electrophoresis (comet assay), following the method described by Li et al. (2008). Unless otherwise stated, molecular biology-grade DNAse-free reagents (Sigma− Aldrich) were used throughout.

54
Mol. Reprod. Dev. 82:48–57 (2015)

- 65 -

Microscope slides (OxiSelectST; Cell Biolabs, Inc.) were prepared in the following manner: A 100 μl sperm sample was diluted in 5 ml of Ca^{2+}- and Mg^{2+}-free phosphate buffer saline (PBS) to obtain a spermatozoa density of 1×10^7 cells/ml. Diluted samples (200 μl) were mixed with 700 μl 0.8% NuSieve GTG low-melting-point agarose (OxiSelectST; Cell Biolabs, Inc.). Finally, 55 μl of this mixture was added to the slide, and the agarose was allowed to solidify for 1 hr at 4°C. The slides were then immersed in lysis buffer (2.5 M NaCl, 100 mM EDTA, 10% Lysis Solution [OxiSelect^{TM} Comet Assay Kit], 10% DMSO [pH 10]) at 4°C.

Slides were subjected to enzyme treatment with proteinase K (1 mg/ml in 2.5 M NaCl, 100 mM EDTA, 10% DMSO [pH 7.4]) and incubated for 12 hr at 32°C. After the proteinase K solution was drained, slides were immersed in a horizontal gel tank filled with freshly mixed electrophoresis buffer (90 mM Tris, 90 mM Boric acid, 2.5 mM EDTA). Electrophoresis was carried out for 20 min at 35 V and 170 mA. Slides were carefully transferred from the electrophoresis chamber to a clean container with pre-chilled distilled water for 2 min; this washing procedure was repeated three times. Slides were drained well, dehydrated by dipping into absolute ethanol for 5 min, and air dried for storage. Prior to analysis, 50 μl of Vista Green DNA Staining Solution (OxiSelectST; Cell Biolabs, Inc., USA) was added to each agarose spot on the slide and examined using an Olympus BX50 fluorescence microscope at 20× magnification. One hundred spermatozoa were scored for each sample, and the captured images were analyzed using CASP software (Comet Assay Software Project Lab: http://casplab.com/). The percentage of DNA in tail was calculated using the following formula:

% Tail DNA = 100 × Tail DNA Intensity/Cell DNA Intensity

Protein Extraction

Proteins were extracted from spermatozoa with lysis buffer (8 M Urea, 2 M Thiourea, 4% CHAPS, 10% w/v isopropanol, 0.1% w/v Triton X-100, 100 mM dithiothreitol) containing phosphatase inhibitors (1 mM sodium orthovanadate, 50 mM EDTA, 1 μM okadaic acid) and protease inhibitors (100 mM PMSF, 1 μg/ml pepstatin A, 5 μg/ml leupeptin). The bicinchoninic acid assay was used to determine the protein concentration in samples.

One-Dimensional SDS-PAGE Separation

For SDS-PAGE, samples (25 μg of proteins per lane) were resuspended in buffer containing 65 mM Tris, 10% (v/v) glycerol, 2% (w/v) SDS, and 5% (v/v) beta-mercaptoethanol, and then heated for 3 min at 95 °C. Proteins were separated on a 12% gel using the Bio-Rad Mini-PROTEAN vertical electrophoresis system.

Two-Dimensional PAGE Separation

Isoelectric focusing was performed on ReadyStrip IPG strips (pH 5−8, 11 cm) with PROTEAN IEF (Bio-Rad). A total of 150 μg of protein in 200 μl of rehydrating buffer (8 M urea, 2 M thiourea, 4% CHAPS, 50 mM dithiothreitol, 0.4% IPG buffer) was applied to each IPG strip. The following conditions were used for separation: active rehydration at 50 V for 14 hr; isoelectric focusing, 500 V for 1 hr, 1000 V for 1 hr, 3,000 V for 1 hr (gradient), and 8,000 V for 2 hr (gradient). After isoelectric focusing, IPG strips were equilibrated with a buffer containing 6 M urea, 29.3% glycerol, 2% SDS, 75 mM Tris−HCl [pH 8.8], and 2% dithiothreitol for 15 min, followed by a second equilibration with a solution containing 2.5% iodacetamide instead of dithiothreitol for a further 15 min. The strip was fixed to a 12% SDS gel with 0.5% agarose in running buffer for SDS-PAGE, and then separated on the specific gel. Protein spots were visualized by staining with Coomassie Brilliant Blue R-250.

Western-Blot Analysis

After electrophoresis, gels were placed on polyvinylidene difluoride (PVDF) membranes (Biorad, Prague, Czech Republic) and electrically transferred. The membranes were blocked by incubation with 5% (w/v) non-fat milk in TBST (0.1% Tween-20, 20 mM Tris, 150 mM NaCl [pH 7.6]) for 1 hr at 20 °C. The membranes were washed three times with TBST, and then incubated for 12 hr at 4°C with anti-phosphotyrosine antibodies (Millipore) diluted 1:500 in 5% bovine serum albumin in TBST (BSA-TBST). Membranes were then washed and incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG (1:3,000 in 3% BSA-TBST) for 1 hr at 20°C. Reacted proteins were revealed with 3,3′,5,5′ tetramethylbenzidine (TMB) liquid substrate (Sigma−Aldrich). Total protein loading was controlled after immunodetection by staining the blot with 0.1% Coomassie Brilliant Blue R-250 in isopropanol. Membranes were destained for 15 min in acetic acid/ethanol/water (1:5:4), washed with water, and finally air-dried.

In-Gel Digestion and Mass Spectrometry

Gels were washed in water, and protein spots were cut from gels. The gel pieces were de-stained by incubation with 50% acetonitrile in 50 mM $(NH_4)HCO_3$ for 15 min. The gel pieces were treated with 5 μl of 50 mM $(NH_4)HCO_3$ containing 1 μg of modified sequencing-grade trypsin (Promega, Madison, WI), and incubated for 12 hr at 37°C. After digestion, the gel pieces were immersed in 100 μl of 0.1% trifluoroacetic acid (TFA) in water for further peptide extraction. The peptides were concentrated and desalted using Zip-Tip pipette tips (Millipore). The Zip-Tip was sequentially washed with 100% acetonitrile, 50% acetonitrile-0.1% TFA, and 0.1% TFA in water. After equilibration of the Zip-Tip, sample was loaded and contaminants were eluted with 0.1% TFA. Peptides were eluted with 2 μl of 50% acetonitrile-0.1% TFA in water. A 5 mg/ml solution of α-cyano-4-hydroxycinnamic acid (α-CHCA) in 50% acetonitrile-0.1% TFA was used as a matrix. One microliter of each sample was spotted on a steel MALDI target plate, and then 1 μl of freshly made α-CHCA matrix

The effect of reactive oxygen species on motility parameters, DNA integrity, tyrosine phosphorylation and phosphatase activity of common carp (Cyprinus carpio L.) spermatozoa

Molecular Reproduction & Development

GAZO *ET AL.*

was added to the sample. This mixture was left to dry at room temperature.

Peptides were analyzed by MALDI-TOF MS/MS using an Autoflex Speed (Bruker Daltonics). Tandem mass spectra were extracted using Xcalibur (Thermo Fisher, v 2.0), and uploaded to a Mascot (Matrix Science, London, UK, v. 2.2) server to search against a NCBInr Cyprinidae database (database containing 99,973 proteins was generated on 2013.02.22) with a fragment ion mass tolerance of 0.8 Da and parent ion mass tolerance of 2 Da.

Acid Phosphatase Activity

Sperm samples for phosphatase extraction were centrifuged to remove IM at 5,000g at 4°C for 10 min. The supernatant was carefully collected and discarded. The spermatozoa pellet was diluted with 20 mM Tris buffer [pH 7.0] containing 1 mM dithiothreitol, and then homogenized in an ice bath using a Sonopuls HD 2070 ultrasonicator (Bandelin Electronic, Berlin, Germany). Extracts were centrifuged at 12,000g for 30 min at 4°C. Acid phosphatase activity was measured after 30 min of incubation with 5 mM p-nitrophenylphosphate (disodium salt) in 20 mM citrate buffer, [pH 5.0] at 37°C; the reaction was terminated with 1 N NaOH. The absorbance was read at 410 nm, and enzymatic activity was expressed as U/L (mM of substrate hydrolyzed per min). To assess the specificity of this acid phosphatase activity assay for tyrosine phosphatase, spermatozoa were incubated for 5 min with sodium orthovanadate (Na_3VO_4), an inhibitor of tyrosine phosphatase.

Data Analysis

All measurements were conducted in triplicate. Normality and the homogeneity of variance of all data were first tested with the Kolmogorov test and the Bartlett test, respectively. Data obtained from the comet assay were log-transformed. Values are expressed as means ± standard deviation (n = 6). Statistical comparison was made by analysis of variance (ANOVA) followed by Tukeys HSD test for spermatozoa velocity and percentage of tail DNA. Spermatozoa motility data and results of the acid phosphatase activity assay were analyzed using the Kruskal−Wallis test. All analyses were performed at a significance cut-off of 0.05 using STATISTICA v. 9.0 software for Windows.

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REFERENCES

Agarwal A, Saleh RA, Bedaiwy MA. 2003. Role of reactive oxygen species in the pathophysiology of human reproduction. Fertil Steril 79:829−843.

Agarwal A, Said TM. 2005. Oxidative stress, DNA damage and apoptosis in male infertility: A clinical approach. BJU Int 95:503−507.

Aitken RJ, Buckingham D, Harkiss D. 1993. Use of a xanthine-oxidase free-radical generating-system to investigate the cytotoxic effects of reactive oxygen species on human spermatozoa. J Reprod Fertil 97:441−450.

Aitken RJ, Paterson M, Fisher H, Buckingham DW, van Duin M. 1995. Redox regulation of tyrosine phosphorylation in human spermatozoa and its role in the control of human sperm function. J Cell Sci 108:2017−2025.

Aitken RJ. 1997. Molecular mechanisms regulating human sperm function. Mol Hum Reprod 3:169−173.

Aitken RJ, Baker MA. 2006. Oxidative stress, sperm survival and fertility control. Mol Cell Endocrinol 250:66−69.

Boitano S, Omoto CK. 1991. Membrane hyperpolarization activates trout sperm without an increase in intracellular pH. J Cell Sci 98:343−349.

Chance B, Sies H, Boveris A. 1979. Hydroperoxide metabolism in mammalian organs. Physiol Rev 59:527−605.

de Lamirande E, Jiang H, Zini A, Kodama H, Gagnon C. 1997. Reactive oxygen species and sperm physiology. Rev Reprod 2:48−54.

Dobbelaere J, Gentry MS, Hallberg RL, Barral Y. 2003. Phosphorylation-dependent regulation of septin dynamics during the cell cycle. Dev Cell 4:345−357.

DuTeaux SB, Berger T, Hess RA, Sartini BL, Miller MG. 2004. Male reproductive toxicity of trichloroethylene: Sperm protein oxidation and decreased fertilizing ability. Biol Reprod 70:1518−1526.

Ford WCL. 2004. Regulation of sperm function by reactive oxygen species. Hum Reprod Update 10:387−399.

Gazo I, Linhartova P, Shaliutina A, Hulak M. 2013. Influence of environmentally relevant concentrations of vinclozolin on quality, DNA integrity, and antioxidant responses of sterlet *Acipenser ruthenus* spermatozoa. Chem Biol Interact 203:377−385.

Hagedorn M, McCarthy M, Carter VL, Meyers SA. 2012. Oxidative stress in zebrafish (*Danio rerio*) sperm. PLoS ONE 7:e39397.

Hecht D, Zick Y. 1992. Selective inhibition of protein tyrosine phosphatase activities by H_2O_2 and vanadate in vitro. Biochem Biophys Res Commun 188:773−779.

Ihara M, Kinoshita A, Yamada S, Tanaka H, Tanigaki A, Kitano A, Goto M, Okubo K, Nishiyama H, Ogawa O, Takahashi C, Itohara S, Nishimune Y, Noda M, Kinoshita M. 2005. Cortical organization by the septin cytoskeleton is essential for structural and mechanical integrity of mammalian spermatozoa. Dev Cell 8:343−352.

Inaba K. 2003. Molecular architecture of the sperm flagella: Molecules for motility and signaling. Zool Sci 20:1043−1056.

Kodoma H, Yamaguchi R, Fukuda J, Kasai H, Tanaka T. 1997. Increased oxidative deoxyribonucleic acid damage in the spermatozoa of infertile male patients. Fertil Steril 68:519−524.

Krasznai Z, Marian T, Izumi H, Damjanovich S, Balkay L, Tron L, Morisawa M. 2000. Membrane hyperpolarization removes in-activation of Ca^{2+} channels, leading to Ca^{2+} influx and subsequent initiation of sperm motility in the common carp. Proc Natl Acad Sci USA 97:2052−2057.

Lewis B, Aitken RJ. 2001. A redox-regulated tyrosine phosphory-lation cascade in rat spermatozoa. J Androl 22:611−622.

Li P, Wei Q, Liu L. 2008. DNA integrity of *Polyodon spathula* cryopreserved sperm. J Appl Ichthyol 24:121−125.

Li P, Hulak M, Li ZH, Sulc M, Psenicka M, Rodina M, Gela D, Linhart O. 2013. Cryopreservation of common carp (*Cyprinus carpio* L.) sperm induces protein phosphorylation in tyrosine and threonine residues. Theriogenology 80:84−89.

Linhartova P, Gazo I, Shaliutina A, Hulak M. 2013. The in vitro effect of duroquinone on functional competence, genomic in-tegrity, and oxidative stress indices of sterlet (*Acipenser ruthenus*) spermatozoa. Toxicol In Vitro 27:1612−1619.

Morisawa M, Suzuki K. 1980. Osmolality and potassium ion: Their roles in initiation of sperm motility in teleosts. Science 210:1145−1147.

Morita M, Nakajima A, Takemura A, Okuno M. 2011. Involvement of redox- and phosphorylation-dependent pathways in osmotic adaptation in sperm cells of euryhaline tilapia. J Exp Biol 214:2096−2104.

OFlaherty C, de Lamirande E, Gagnon C. 2006. Reactive oxygen species modulate independent protein phosphorylation path-ways during human sperm capacitation. Free Radic Biol Med 40:1045−1055.

Perchec G, Jeulin C, Cosson J, Andre F, Billard R. 1995. Rela-tionship between sperm ATP content and motility of carp sper-matozoa. J Cell Sci 108:747−753.

Ray PD, Huang BW, Tsuji Y. 2012. Reactive oxygen species (ROS) homeostasis and redox regulation in cellular signaling. Cell Signal 24:981−990.

Rodina M, Gela D, Kocour M, Alavi SMH, Hulak M, Linhart O. 2007. Cryopreservation of tench, *Tinca tinca*, sperm: Sperm motility and hatching success of embryos. Theriogenology 67: 931−940.

Sanocka D, Kurpisz M. 2004. Reactive oxygen species and sperm cells. Reprod Biol Endocrinol 2:12.

Sarosiek B, Pietrusewicz M, Radziwoniuk J, Glogowski J. 2009. The effect of copper, zinc, mercury and cadmium on some sperm enzyme activities in the common carp (*Cyprinus carpio* L.). Reprod Biol 9:295−301.

Shaliutina A, Hulak M, Gazo I, Linhartova P, Linhart O. 2013. Effect of short-term storage on quality parameters, DNA integrity, and oxidative stress in Russian (*Acipenser gueldenstaedtii*) and Siberian (*Acipenser baerii*) sturgeon sperm. Anim Reprod Sci 139:127−135.

Spiliotis ET, Nelson WJ. 2006. Here come the septins: Novel polymers that coordinate intracellular functions and organiza-tion. J Cell Sci 119:4−10.

Spiliotis ET. 2010. Regulation of microtubule organization and functions by septin GTPases. Cytoskeleton (Hoboken) 67:339−345.

Visconti PE, Moore GD, Bailey JL, Leclerc P, Connors SA, Pan D, Olds-Clarke P, Kopf GS. 1995. Capacitation of mouse sperma-tozoa. II. Protein tyrosine phosphorylation and capacitation are regulated by a cAMP-dependent pathway. Development 121:1139−1150.

Wang X, Sharma RK, Gupta A, George V, Thomas AJ, Falcone T, Agarwal A. 2003. Alterations in mitochondria membrane poten-tial and oxidative stress in infertile men: A prospective observa-tional study. Fertil Steril 80:844−850.

Whelan SA, Lane MD, Hart GW. 2008. Regulation of the O-linked beta-N-acetylglucosamine transferase by insulin signaling. J Biol Chem 283:21411−21417.

Wong-Ekkabut J, Xu Z, Triampo W, Tang IM, Tieleman DP, Monticelli L. 2007. Effect of lipid peroxidation on the properties of lipid bilayers: A molecular dynamics study. Biophys J 93:4225−4236.

Wu L, Sampson NS. 2014. Fucose, mannose, and β-N-Acetyl-glucosamine glycopolymers initiate the mouse sperm acrosome reaction through convergent signaling pathways. ACS Chem Biol 9:468−475.

Zachara NE, ODonnell N, Cheung WD, Mercer JJ, Marth JD, Hart GW. 2004. Dynamic O-GlcNAc modification of nucleocytoplas-mic proteins in response to stress. A survival response of mammalian cells. J Biol Chem 279:30133−30142.

Zeidan Q, Hart GW. 2010. The intersections between O-GlcNA-cylation and phosphorylation: implications for multiple signaling pathways. J Cell Sci 123:13−22.

Zhou BS, Liu WH, Siu WHL, OToole D, Lam PKS, Wu RSS. 2006. Exposure of spermatozoa to duroquinone may impair reproduc-tion of the common carp (*Cyprinus carpio*) through oxidative stress. Aquat Toxicol 77:136−142.

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57

MOLECULAR MECHANISMS OF MOTILITY ACTIVATION AND INTRACELLULAR SIGNALING IN SPERMATOZOA OF TWO TAXONOMICALLY DISTANT FISH SPECIES: STERLET (*ACIPENSER RUTHENUS*) AND COMMON CARP (*CYPRINUS CARPIO* L.)

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SUMMARY

Spermatozoa of externally fertilizing freshwater fish possess different modes of motility activation. Spermatozoa of common carp (Cyprinus carpio L.) are activated by hypoosmolality, whereas spermatozoa of sterlet (Acipenser ruthenus) require Ca^{2+} and low concentration of K^+ for motility activation. Furthermore, intracellular signaling differs between these two species as well. The current study was performed in order to reveal molecular mechanisms of motility activation, roles of protein phosphorylation and protein kinases in spermatozoa of carp and sterlet. Our results indicate that protein kinases A and C (PKA and PKC) participate in spermatozoa motility of both species. Immunolabeling of phospho-(Ser/Thr) PKA substrates indicate that phosphorylated proteins are localized differently in spermatozoa of carp and sterlet. Strong phosphorylation of PKC substrate was observed in flagella of sterlet spermatozoa, whereas in carp sperm PKC substrates were lightly phosphorylated in the midpiece and flagella. Motility activation also induced phosphorylation / dephosphorylation on serine, threonine and tyrosine residues of numerous proteins in carp and sterlet spermatozoa. Proteomic methods were used to identify phospho-proteins which participate in sperm motility. Numerous mitochondrial and glycolytic enzymes were identified in spermatozoa of both species, as well as axonemal proteins and signaling enzymes involved in sperm motility. These results contribute to an understanding of the role of signaling molecules, protein kinases and protein phosphorylation in motility activation and regulation of two valuable fish species, C. carpio and A. ruthenus.

Keywords: Spermatozoa motility, phosphorylation, protein kinases, intracellular signaling

INTRODUCTION

Sperm motility represents a very important prerequisite leading to the contact and fusion between male and female gametes at fertilization. There are different triggers of sperm motility activation in externally fertilizing species, such as osmotic changes, differences in ion concentrations between seminal plasma and external media, sperm-activating peptides, and chemoattractants. In some freshwater fish species, such as carp (*Cyprinus carpio*), very low ambient water osmolality is directly involved in spermatozoa motility activation (Perchec et al., 1997), whereas in sturgeons activation can be achieved without osmolality alteration (as compared with seminal fluid) through changes in ionic composition of the surrounding medium (Alavi and Cosson, 2006). Furthermore, mechanisms of intracellular signaling vary

significantly among different species. Even shape of spermatozoa differs between species. Thus carp spermatozoa possess a round-shape head without an acrosome. While in sturgeons the sperm head is elongated and displays an acrosome at the anterior part (Psenicka et al., 2007). The one common feature for all fish spermatozoa is their transcriptional inactivation, which means there is no protein synthesis in spermatozoa after maturation. Thus one of the most important processes in sperm intracellular signaling is protein post-translational modification. Protein phosphorylation is a type of post-translational modification that has been extensively studied in spermatozoa (O'Flaherty et al., 2006).

The phosphorylation of proteins on serine/threonine (Ser/Thr) and tyrosine (Tyr) residues is controlled by the activity of protein kinases, and the dephosphorylation of these residues by protein phosphatases. Thus, the balance of the opposing activities of these enzymes determines the majority of intracellular signaling pathways (Aitken et al., 1995; Ray et al., 2012).

Processes regulated by protein phosphorylation include motility activation, capacitation, and the acrosome reaction in mammals, all of which are required for spermatozoa to reach and fuse with the oocyte (O'Flaherty et al., 2006). The number of structures in spermatozoa axoneme is known to be regulated by phosphorylation / dephosphorylation. Most of those proteins in fish are still to be characterized, but some phosphoproteins participating in sperm motility have been described (Inaba et al., 1998; Itoh et al., 2003). Thus it has been shown that dynein is one of the major targets of cAMP-dependent protein phosphorylation (Hamasaki et al., 1991). Dyneins are microtubule-dependent force-generating ATPases and play a key role in microtubule sliding (Visconti et al., 1995; Inaba, 2003). Additionally there is a strong correlation between Ca^{2+} signaling and protein phosphorylation in sperm flagella. Axonemes contain several Ca^{2+} binding proteins with EF-hands, such as a light chain of outer arm dynein, a subunit of dynein docking complex, and calmodulin (CaM) in radial spoke (Inaba, 2007).

Energy metabolism is a key factor supporting sperm function. Oxidative phosphorylation (OXPHOS) is an active pathway of sperm energy production in many species (Storey, 2008). Moreover numerous phosphorylated proteins were identified within the mitochondria and on signaling pathways that act on mitochondria (Hopper et al., 2006; Huttemann et al., 2007; Pagliarini and Dixon, 2006).

On the other hand, Nascimento et al. (2008) showed that for motile human spermatozoa, the glycolytic pathway is a primary source of energy. Unlike OXPHOS that takes place only in the midpiece, glycolytic enzymes are localized all along the flagellum allowing more efficient transport of ATP to dynein ATPases (Krisfalusi et al., 2006). Studies on mammalian spermatozoa indicate an activation of glycolytic process in response to increased cAMP levels, suggesting the interplay between sperm motility initiation and activation of glycolysis (Frenkerl et al., 1973). In fish spermatozoa the role of the glycolytic pathway of energy production was described for *Clarias gariepinus* (Mansour et al., 2003).

The phosphorylated state of proteins is controlled by various kinases, which regulate spermatozoa intracellular signaling. The contribution of the cAMP-dependent protein kinase A (PKA) as a regulator of spermatozoa motility was described for numerous species (Inaba, 2003; Brokaw, 1987). PKA is anchored near the outer arm dynein, where it is regulated by proteasomes, and is involved in phosphorylation of axonemal proteins (Inaba et al., 1998). On the other hand PKA is also involved in signaling processes on the outer side of the mitochondria, and can translocate into the mitochondria to phosphorylate proteins directly (Huttemann et al., 2007).

The cAMP-independent phosphorylation of axonemal proteins during spermatozoa motility initiation has been reported as well (Hayashi et al., 1987; Morita et al., 2003). It has been shown that in carp spermatozoa motility activation is induced by hypoosmolality and this

process is cAMP independent (Krasznai et al., 2000). Spermatozoa of taxonomically distant sturgeons however require cAMP and Ca^{2+} ions for motility activation, thus representing a totally different mode of sperm motility activation (Alavi et al., 2011; Linhart et al., 2002).

Thus it is known that extracellular factors controlling sperm motility act on the flagellar motile apparatus, the axoneme, through signal transduction, where cAMP and Ca^{2+} serve as second messengers. Second messengers determine the sperm motility initiation by modification of dynein-mediated sliding of the axonemal outer-doublet microtubules through protein phosphorylation/dephosphorylation in different mammalian species (Lindemann and Kanous, 1989), rainbow trout, chum salmon, sea urchin (Inaba et al., 1999), and tunicate (Nomura et al., 2000). However, there is a lack of knowledge about the role of protein phosphorylation in spermatozoa of commercially important, aquaculture fish species such as sterlet (*Acipenser ruthenus*) and common carp (*Cyprinus carpio* L.). Therefore the present study was undertaken in order to provide essential knowledge about the molecular mechanisms of motility activation, roles of protein phosphorylation and protein kinases in spermatozoa of these two taxonomically distant fish species. Specifically, we wish to determine how the protein phosphorylation pattern changed after motility activation in carp and sterlet spermatozoa, where phosphorylated proteins are located in the spermatozoon and to identify proteins involved in sperm motility.

MATERIALS AND METHODS

Chemicals

Inhibitors of protein kinases: staurosporine, H-89, chelerythrine, Gö6983 and tyrphostin 23 were purchased from the Sigma-Aldrich Co. (St Louis, LO, USA). ReadyStrip IPG strips, protein marker and polyvinylidene difluoride (PVDF) membranes were from Bio-Rad laboratories (San Francisco, CA, USA). Anti-phosphotyrosine, anti-phosphoserine and anti-phosphothreonine polyclonal antibodies produced in rabbit were from Abcam (Cambridge, UK). Phospho-(Ser/Thr) PKA substrate, phospho-(Ser) PKC substrate polyclonal antibodies produced in rabbit and HRP-conjugated goat anti-rabbit IgG were purchased from Cell Signaling Technology (Biotech A.S., Prague, Czech Republic).

Broodstock handling and sperm collection

All experiments were performed according to the principles of the Ethical Committee for the Protection of Animals in Research of the University of South Bohemia, Research Institute of Fish Culture and Hydrobiology, Vodnany, Czech Republic. Sperm was obtained from adult common carp (n = 6; body weight, 2–3 kg) and sterlet (n = 6; body weight, 0.5–2 kg) reared in the aquaculture facility of the Research Institute of Fish Culture and Hydrobiology at the University of South Bohemia, Vodnany, Czech Republic. Until experimentation, fish were held in tanks at water temperature of 22 °C for carp and 14–15 °C for sterlet. Spermiation was stimulated by intramuscular injection of carp pituitary powder dissolved in 0.9% (w/v) NaCl solution at 2 mg kg^{-1} body weight for carp and 5 mg kg^{-1} of body weight for sterlet, 24 hr prior to stripping. Sperm samples from carp were obtained by abdominal massage and collected directly into 10 ml plastic syringes. Sterlet semen was collected from the urogenital papilla by aspiration through a plastic catheter (5–7 mm diameter) connected to a 20 ml syringe. Care was taken to avoid contamination with mucus, faeces, or water. Samples were stored on ice (0–4 °C) in closed assay tubes prior to analysis. Spermatozoa concentration of each male was estimated microscopically (Olympus BX 41) at 20× using a Burker cell hemocytometer. Average sperm concentration was $55.1 \pm 2.2 \times 10^9$/ml for carp and $1.26 \pm 0.7 \times 10^9$/ml for sterlet.

Sample Preparation

Separate sperm samples from each experimental male were centrifuged at 300g for 30 min at 4 °C to remove seminal plasma. The resulting pellets were diluted in an immobilizing medium (IM). IM for carp was prepared according to (Perchec et al., 1995) and contained 200 mM KCl, 30 mM Tris-HCl, pH 8.0; IM for sterlet contained 20 mM Tris, 30 mM NaCl, 2 mM KCl, pH 8.5. Sperm was diluted to a final concentration of 5×10^8 cells/ml. Protein kinase inhibitors were first dissolved in dimethyl sulfoxide at 100 mM and then dissolved to desired concentrations with IM, so that the final concentration of DMSO in incubation medium was 0.1%. The sperm sub-samples were incubated at 4 °C for 3 min with following inhibitors: staurosporine (broad spectrum inhibitor of protein kinases), H-89 (PKA inhibitor), chelerythrine and Gö6983 (inhibitors of PKC) and tyrphostin 23 (PKT inhibitor). After incubation spermatozoa motility was recorded and analysed.

Samples for western blotting and protein analysis were prepared as follows: sperm pellets were diluted either with IM or with activation medium (AM) containing 5 mM KCl, 45 mM NaCl, 30 mM Tris, pH 8.2 for carp sperm; and 10 mM Tris, 10 mM NaCl, 1 mM CaCl$_2$, pH 8.5 for sterlet at a dilution ratio of 1:50. Sperm samples were frozen in liquid nitrogen at different time points after motility activation (0, 10, 30, 60 s for carp; 0, 30, 60, 120 s for sterlet) and stored at -80 °C.

Spermatozoa motility and velocity analysis

The percentage of motile spermatozoa was determined after triggering sperm motility under dark-field microscopy (Olympus BX 50, Japan). For triggering motility, sperm was diluted in AM at a dilution of 1:5000. To prevent spermatozoa from sticking to the microscope slide, 0.25% (w/v) pluronic acid was included in AM. Spermatozoa motility was recorded with a CCD video camera (SONY SSCDC50AP, Japan) mounted on a microscope, using 20× magnification objective lens and illumination by a stroboscopic flash (ExposureScope™). The positions of the heads of spermatozoa were measured from five successive frames and analysed with a micro-image analyser (Olympus Micro Image 4.0.1. for Windows). Motility was assessed at 15 s post-activation as described by Rodina et al. (2007). Measurement of spermatozoa motility for each sample was performed in triplicate.

Protein extraction

Proteins were extracted from spermatozoa with lysis buffer (8M Urea, 2M Thiourea, 4% CHAPS, 10% w/v isopropanol, 0.1% w/v Triton X-100, 100 mM dithiothreithol) containing phosphatase inhibitors (1mM sodium orthovanadate, 50mM EDTA, 1mM okadaic acid) and protease inhibitors (100mM PMSF, 1mg/ml pepstain A, 5 mg/ml leupeptin). The bicinchoninic acid assay was used to determine the protein concentration in samples.

One-Dimensional SDS-PAGE Separation

For SDS-PAGE, samples (25 µg of proteins per lane) were resuspended in buffer containing 65 mM Tris, 10% (v/v) glycerol, 2% (w/v) SDS, and 5% (v/v) beta-mercaptoethanol, and then heated for 3 min at 95 °C. Proteins were separated on a 12% gel using the Bio-Rad Mini-PROTEAN vertical electrophoresis system.

Two-Dimensional IEF + PAGE Separation

For two-dimensional (2D) gel separation proteins extracted from immotile sterlet and carp spermatozoa were used as well as proteins from spermatozoa at 30 s post-activation for carp sperm and 120 s for sterlet. Isoelectric focusing was performed on ReadyStrip IPG strips (pH 3–10, 7 and 11 cm) with PROTEAN IEF (Bio-Rad). A total of 150 µg of protein in

200 μl of rehydrating buffer (8M Urea, 2M Thiourea, 4% CHAPS, 50mM dithiothreitol, 0.4% IPG buffer) was applied to each IPG strip. The following conditions were used for separation: active rehydration at 50 V for 14 hr; isoelectric focusing, 500 V for 1 hr, 1,000 V for 1 hr, 3,000 V for 1 hr (gradient), and 8,000 V for 2 hr (gradient). After isoelectric focusing, IPG strips were equilibrated with a buffer containing 6M Urea, 29.3% glycerol, 2% SDS, 75 mM Tris–HCl [pH 8.8], and 2% dithiothreitol for 15min, followed by a second equilibration with a solution containing 2.5% iodacetamide instead of dithiothreitol for a further 15min. The strip was fixed toloaded on a 12% SDS gel with 0.5% agarose in running buffer for SDS-PAGE, and then the proteins separated according to molecular weight on by electrophoresisthe specific gel. Protein spots were visualized by staining with Coomassie Brilliant Blue R-250.

Western-Blot Analysis

After electrophoresis, gels were placed on polyvinylidene difluoride (PVDF) membranes and electrically transferred. The membranes were blocked by incubation with 5% (w/v) non-fat milk in TBST (0.1% Tween-20, 20 mM Tris, 150 mM NaCl [pH 7.6]) for 1 hr at 20 °C. The membranes were washed three times with TBST, and then incubated for 12 hr at 4 °C with polyclonal antibodies to phospho-tyrosine, phospho-serine and phospho-threonine or to phospho-(Ser/Thr) PKA and phospho-(Ser) PKC substrates diluted 1:1000 in 5% bovine serum albumin in TBST (BSA-TBST). Membranes were then washed and incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG (1:3000 in 3% BSA-TBST) for 1 hr at 20 °C. Reacted proteins were detected either with 3,3′,5,5′ tetramethylbenzidine (TMB) liquid substrate or with ECL-plus. Total protein loading was controlled after immunodetection by staining the blot with 0.1% Coomassie Brilliant Blue R-250 in isopropanol. Membranes were destained for 15min in acetic acid/ethanol/water (1:5:4), washed with water, and finally air-dried.

Immunolabeling

Sperm motility was activated by dilution with activation medium at a dilution ratio of 1:500. Spermatozoa were attached on slides pre-coated with 0.01% poly-L-lysine. After incubation for 5 min at 20 °C, the sperm were fixed with 4% formaldehyde in IM for 30 min and stored in a moist chamber. Spermatozoa were rehydrated and permeabilized using TBST. The slides were incubated with blocking buffer (3% BSA-TBST) for 1 h at 20 °C and then with primary antibody to phospho-tyrosine, phospho-serine and phospho-threonine or to phospho-(Ser/Thr) PKA and phospho-(Ser) PKC substrates (1:100) in the blocking buffer for 14 h at 4 °C, followed by washing with TBST (5 min) at 20 °C. Samples were then incubated with Rhodamine Red-X-conjugated goat anti-rabbit IgG at 1:200 dilution ratio for 2 h at 20 °C. As a final step, samples were washed with TBST (5 min) at 20 °C, treated with TBST containing 1 μM 4,6-diamidino-phenylindole (DAPI) and mounted in 50% glycerol. Slides were examined by fluorescent microscopy (Olympus BX50) at 60 × magnification.

In-Gel Digestion and Mass Spectrometry

Gels were washed in water, and protein spots were cut from gels. The gel pieces were de-stained by incubation with 50% acetonitrile in 50 mM $(NH_4)HCO_3$ for 15 min. The gel pieces were treated with 5 ml of 50 mM $(NH_4)HCO_3$ containing 1 μg of modified sequencing-grade trypsin, and incubated for 12 hr at 37 °C. After digestion, the gel pieces were immersed in 100 μl of 0.1% trifluoroacetic acid (TFA) in water for further peptide extraction. The peptides were concentrated and desalted using Zip-Tip pipette tips (Millipore, Billerica, MA, USA). The Zip-Tip was sequentially washed with 100% acetonitrile, 50% acetonitrile-0.1% TFA, and 0.1% TFA in water. After equilibration of the Zip-Tip, sample was loaded and contaminants were eluted with 0.1% TFA. Peptides were eluted with 2 μl of 50% acetonitrile-0.1% TFA in water.

A 5 mg/ml solution of α-cyano-4-hydroxycinnamic acid (α-CHCA) in 50% acetonitrile-0.1% TFA was used as a matrix. One microliter of each sample was spotted on a steel MALDI target plate, and then 1 μl of freshly made α-CHCA matrix was added to the sample. This mixture was left to dry at room temperature. Peptides were analysed by MALDI-TOF MS/MS using an Autoflex Speed (Bruker Daltonics). Tandem mass spectra were extracted using Xcalibur (Thermo Fisher, v 2.0), and uploaded to a Mascot (Matrix Science, London, UK, v. 2.2) server to search against a NCBInr database with a fragment ion mass tolerance of 0.8 Da and parent ion mass tolerance of 500 ppm.

RESULTS

Effect of kinase inhibitors on motility of carp and sterlet sperm

The effect of kinase inhibitors on percent motile spermatozoa was assessed at 15 s post-activation (Table 1). In the presence of broad spectrum kinase inhibitor, staurosporine, motility rate of spermatozoa of both species decreased in a dose-dependent manner compared to the control. However, susceptibility of sterlet spermatozoa to staurosporine was higher, compared to carp spermatozoa (at 100 μM: 7.89% motile spermatozoa in sterlet; 66.6% – in carp).

The inhibitor of tyrosine kinase, tyrphostin 23, and the inhibitor of PKC, Gö6983, had no significant effect on spermatozoa motility in both species at all tested concentrations. On the other hand, treatment with an inhibitor of PKC translocation, chelerythrine, inhibited motility of both species at 100 μM, and significantly decreased motility of carp spermatozoa at 10 μM. Motility of carp spermatozoa was affected by the inhibitor of PKA (H-89) at 50 μM; however, motility of sterlet spermatozoa decreased significantly only at 500 μM.

Table 1. The effect of kinase inhibitors on spermatozoa of sterlet (*Acipenser ruthenus*) and carp (*Cyprinus carpio* L.). Data represent mean values ± SD. Different letters indicate statistical significance among samples (ANOVA, P < 0.05).

| Inhibitor	Function	Tested concentrations	Sterlet Motility (%)	Carp Motility (%)
Control (IM, 0.1% DMSO)			80,977 ± 6,31 [A]	80,366 ± 9,486 [A]
Staurosporine	Broad spectrum inhibitor, ATP-site competitor	100 μM	7,894737± 1,165 [D]	66,565 ± 9,67 [B]
		50 μM	67,845 ± 5,034 [B]	68,645 ± 4,074 [AB]
		10 μM	72,58772 ± 8,37 [AB]	71,914 ± 9,575 [AB]
Tyrphostin 23	Receptor type PTK	100 μM	76,875 ± 0,29 [AB]	68,546 ± 9,703 [AB]
		50 μM	78,149 ± 5,68 [A]	67,66 ± 5,54 [AB]
		10 μM	80,5248 ± 4,67 [A]	74,695 ± 4,83 [AB]
Go 6983	Ca^{2+}-dependent PKC α1 and β1	100 μM	75,278 ± 0,39 [AB]	68,528 ± 9,192 [AB]
		50 μM	77,839 ± 7,94 [A]	69,1377 ± 6,4 [AB]
		10 μM	79,1 ± 6,94 [A]	70,83 ± 5,89 [AB]
Chelerythrine	PKC translocation	100 μM	26,875 ± 3,86 [C]	3,545 ± 1,089 [F]
		50 μM	32,23 ± 10,325 [C]	7,987 ± 1,8 [E]
		10 μM	67,903 ± 13,36 [B]	58,0199 ± 8,959 [C]
H-89	ATP-site competitor for PKA	500 μM	68,466 ± 4,59 [B]	
		200 μM	74,38 ± 9,029 [AB]	32,46 ± 3,8 [D]
		100 μM	83,58 ± 0,53 [A]	58,169 ± 11,678 [C]
		50 μM		59,145 ± 13,657 [BC]
		10 μM		70,283 ± 4,074 [AB]

Change of protein phosphorylation pattern after sperm motility activation

Results of western blotting showed that the phosphorylation pattern on three amino acid residues (serine, threonine and tyrosine) changed after motility activation in both species (Fig. 1–3). Incubation with phosphoserine antibodies (Fig. 1) revealed 9 protein bands with molecular weight of 200, 80, 65, 60, 49, 45, 43, 30 and 15 kDa to be phosphorylated/ dephosphorylated after motility activation in spermatozoa of carp. In sterlet spermatozoa 10 protein bands of 120, 90, 70, 60, 55, 52, 40, 30, 27 and 25 kDa changed their phosphorylation state.

The next western blot analysis with antibodies to phosphotyrosine residues revealed that 6 protein bands of 80, 68, 59, 48, 39 and 25 kDa changed phosphorylation state in carp spermatozoa (Fig. 2). A total of 8 proteins of 125, 68, 60, 50, 48, 40, 25and 20 kDa were dephosphorylated in sterlet spermatozoa.
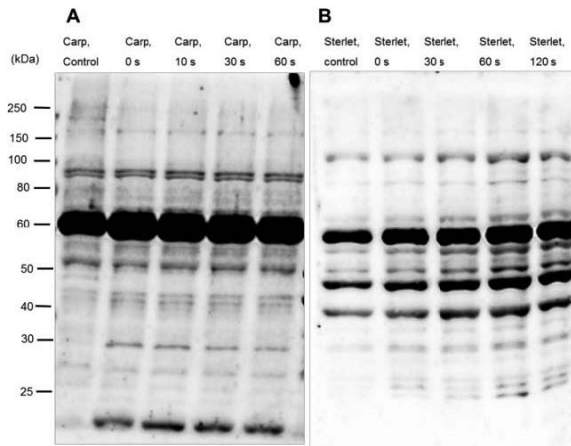
Figure 1. *Western blot analysis with anti-phosphoserine antibody of common carp (A) and sterlet (B) spermatozoa proteins separated using SDS-PAGE. Control indicates control group in immobilizing medium; 0–60 s in carp and 0–120 s in sterlet indicates different time points after motility activation. The number on the left indicates molecular weight marker.*
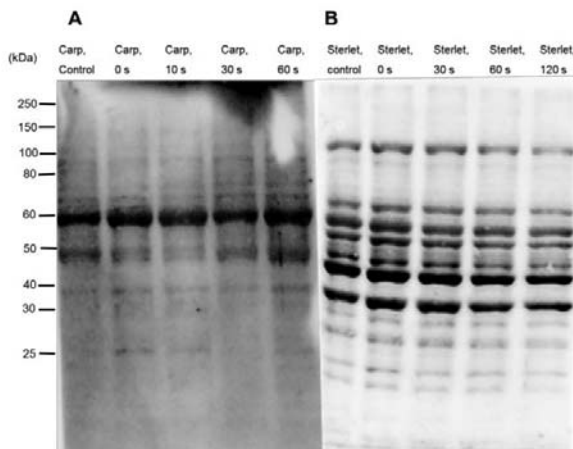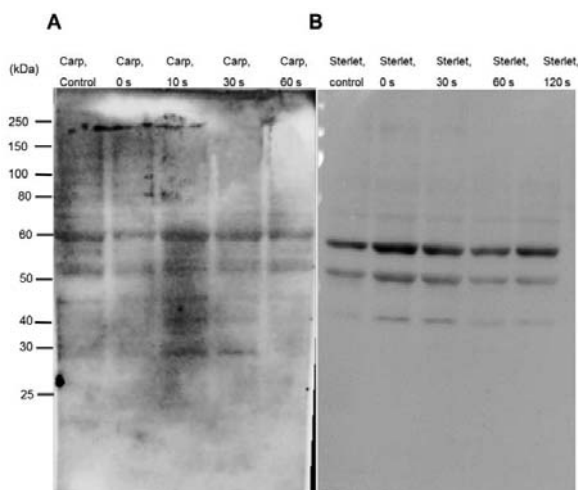

Figure 2. *Western blot analysis with anti-phosphotyrosine antibody of common carp (A) and sterlet (B) spermatozoa proteins separated using SDS-PAGE. Control indicates control group in immobilizing medium; 0–60 s in carp and 0–120 s in sterlet indicates different time points after motility activation. Molecular weight marker is on the left.*

In spermatozoa of common carp 3 protein bands of 55, 45, and 27 kDa changed their phosphorylation state on threonine residue after motility activation (Fig. 3). In sterlet spermatozoa motility activation led to phosphorylation of 59, 52 and 45 kDa proteins. Moreover, phosphorylation on 45 and 27 kDa proteins in carp spermatozoa and 45 kDa band in sterlet appeared to undergo a cyclical transition. These protein bands were phosphorylated up to 30 s after motility activation with subsequent dephosphorylation at the end of motility period.
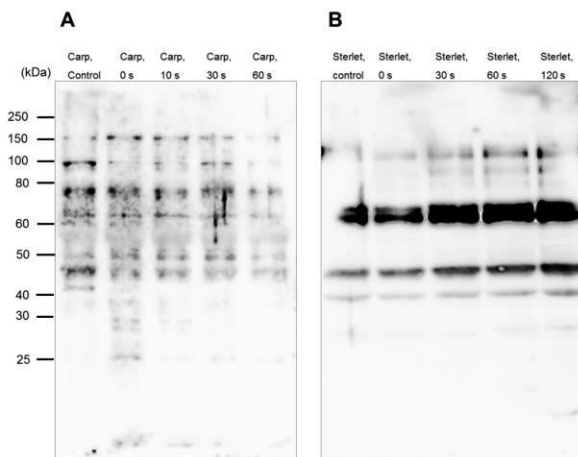
Incubation with phospho-(Ser/Thr) PKA substrate antibodies (Fig. 4) revealed that 8 9 protein bands of 150, 100, 79, 62, 50, 46, 40, 30 and 25 kDa changed their phosphorylation state after motility activation in spermatozoa of carp. In spermatozoa of sterlet 5 PKA substrates protein bands with molecular weight of 150, 120, 45, 42 and 27 kDa changed

phosphorylation state of PKA substrates. Similar to threonine phosphorylation, some PKA substrates in carp sperm (150, 50, 40, 30, 25 kDa) were modified in cyclical manner, with increased phosphorylation at the beginning of motility period and dephosphorylation in the end of motility.
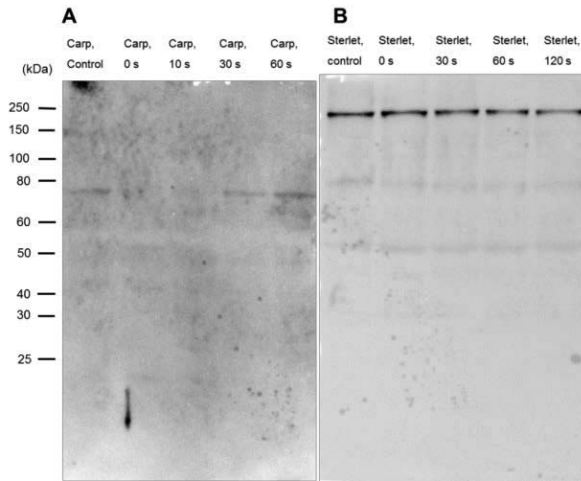
The antibody to phospho-(Ser) PKC substrates (Fig. 5) revealed that one band of 78 kDa was weakly phosphorylated in immotile carp spermatozoa and again after 30 s of motility period. In sterlet spermatozoa a protein band of 200 kDa was phosphorylated in immotile spermatozoa and during all the entire motility period.

Figure 3. *Western blot analysis with anti-phosphothreonine antibody of common carp (A) and sterlet (B) spermatozoa proteins separated using SDS-PAGE. Control indicates control group in immobilizing medium; 0–60 s in carp and 0–120 s in sterlet indicates different time points after motility activation. The number on the left indicates molecular weight marker.*


Figure 4. *Western blot analysis with phospho-(Ser/Thr) PKA substrate antibody of common carp (A) and sterlet (B) spermatozoa proteins separated using SDS-PAGE. Control indicates control group in immobilizing medium; 0–60 s in carp and 0–120 s in sterlet indicates different time points after motility activation. Molecular weight marker is on the left.*

Figure 5. *Western blot analysis with phospho-(Ser) PKC substrate antibody of common carp (A) and sterlet (B) spermatozoa proteins separated using SDS-PAGE. Control indicates control group in immobilizing medium; 0–60 s in carp and 0–120 s in sterlet indicates different time points after motility activation. Molecular weight marker is on the left.*

**Subcellular localization of phospho-proteins in carp and sterlet spermatozoa**

Localization of proteins with phosphorylated serine residue using immuolabeling and fluorescent microscopy showed that in both species prominent phosphorylation was observed in sperm flagella and head (Fig. 6). In carp spermatozoa motility activation led to enhanced serine phosphorylation at the head of spermatozoa.
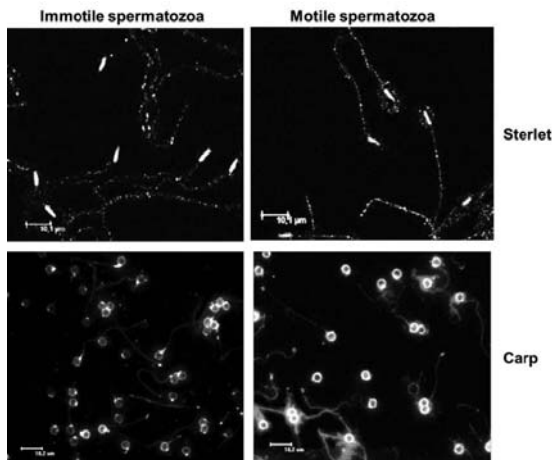
Figure 6. *Immunolocalization of the proteins phosphorylated on serine residue in sterlet and carp spermatozoa. Immotile and activated spermatozoa were fixed on glass slides and treated with anti-phosphoserine antibody, followed by treatment with Rhodamine-conjugated secondary antibodies. Bars represent 10 μm.*
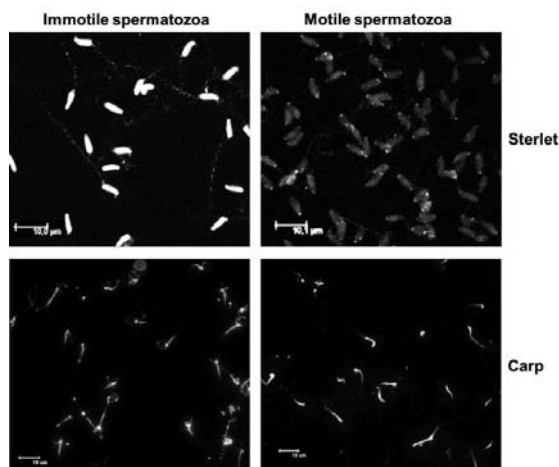
Immunolocalisation of phospho-tyrosine residue showed that in immotile sterlet spermatozoa tyrosine phosphorylation occured in a gradient manner from the head to the tip of the tail (Fig. 7). In carp spermatozoa however, tyrosine phosphorylation occured along the

flagella after motility activation, and in immotile spermatozoa the majority of phosphorylated proteins were localized in the head of spermatozoon.
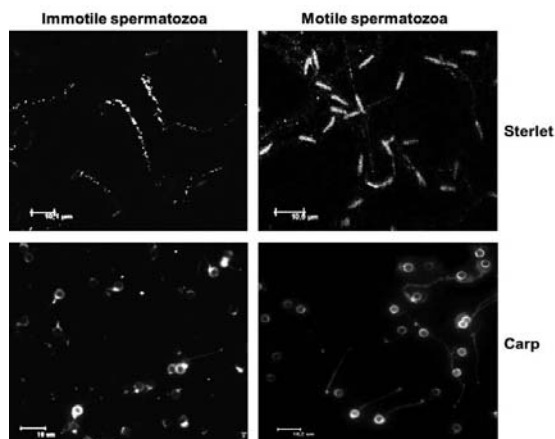
Figure 7. *Immunolocalization of the proteins phosphorylated on tyrosine residue in sterlet and carp spermatozoa. Immotile and activated spermatozoa were fixed on glass slides and treated with anti-phosphotyrosine antibody, followed by treatment with Rhodamine-conjugated secondary antibodies. Bars represent 10 μm.*

Incubation with anti-phosphothreonine antibodies revealed a weak phosphorylation of flagella in sterlet spermatozoa (Fig. 8) that corresponds to results of western blotting (Fig. 3). Most of phosphorylated proteins were localized in the head of immotile spermatozoa and in the midpiece of motile spermatozoa. Immotile carp spermatozoa were phosphorylated on threonine residue mostly in the head and midpiece of spermatozoon (Fig. 8). Phosphorylation on threonine residue translocated to the principal part of flagella after motility activation.

Figure 8. *Immunolocalization of the proteins phosphorylated on threonine residue in sterlet and carp spermatozoa. Immotile and activated spermatozoa were fixed on glass slides and treated with anti-phosphothreonine antibody, followed by treatment with Rhodamine-conjugated secondary antibodies. Bars represent 10 μm.*

Phospho-(Ser/Thr) PKA substrates were localized in flagella and head of immotile sterlet spermatozoa (Fig. 9). After motility activation these phospho-proteins were observed on the sperm membrane. In immotile carp spermatozoa PKA substrates were localized in the midpiece and appeared in flagella only after motility activation.
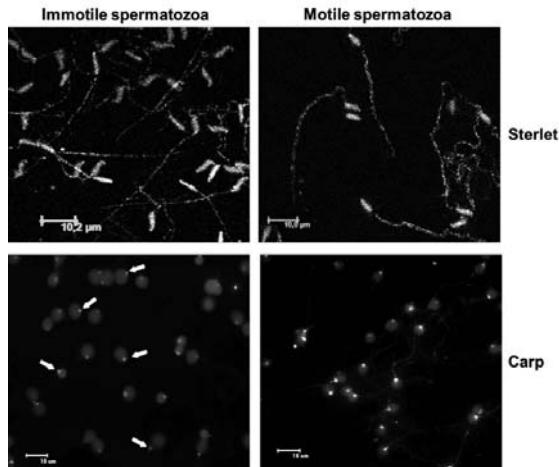
Figure 9. *Immunolocalization of the phospho-PKA substrates in spermatozoa of sterlet and carp. Immotile and activated spermatozoa were fixed on glass slides and treated with anti- phospho-(Ser/Thr) PKA substrate antibody, followed by treatment with Rhodamine-conjugated secondary antibodies. Sperm DNA was stained with DAPI in carp spermatozoa. Arrows indicate PKA substrate phosphorylation in the midpiece of immotile carp spermatozoa. Bars represent 10 μm.*

We observed phosphorylation of sterlet sperm flagella and midpiece proteins with phospho-(Ser) PKC substrate antibodies (Fig. 10). A similar phosphorylation pattern was observed with the same antibodies in carp spermatozoa, however, the intensity of phosphorylation was lower.
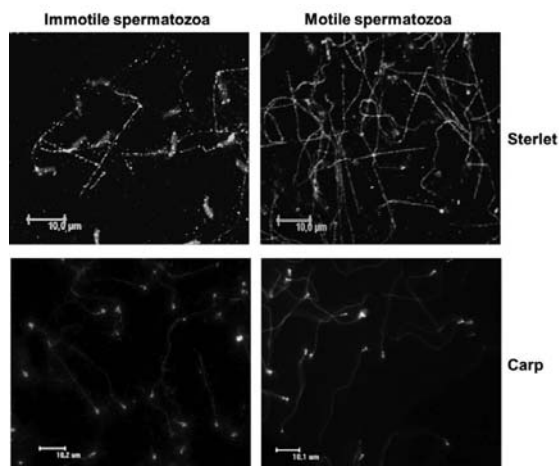

Figure 10. *Immunolocalization of the phospho-PKC substrates in spermatozoa of sterlet and carp. Immotile and activated spermatozoa were fixed on glass slides and treated with anti- phospho-(Ser) PKC substrate antibody, followed by treatment with Rhodamine-conjugated secondary antibodies. Bars represent 10 μm.*

Identification of proteins whose phosphoryation state is modified following motility activation

To identify proteins involved in spermatozoa motility, proteins extracted from control spermatozoa and spermatozoa after motility activation were separated with 2DE, blotted on PVDF membrane, and incubated with phosphoserine, phosphotyrosine, phosphothreonine, phospho-(Ser/Thr) PKA substrate and phospho-(Ser) PKC substrate antibodies (not shown). Fig. 11 represents total protein profiles of sterlet and carp spermatozoa in IM and after motility activation (at 30 s post-activation for carp sperm and 120 s for sterlet). Spots 1–5 in sterlet spermatozoa and spots 1–4 in carp spermatozoa were identified as phospho-(Ser/Thr) PKA substrate, phosphorylated after motility activation. Spot 6 in sterlet spermatozoa was found to be a phospho-(Ser) PKC substrate, slightly dephosphorylated after motility activation. Spots 7–9, 11, 13, 18 in sterlet spermatozoa were dephosphorylated on tyrosine residue after motility activation, whereas spots 10, 12, 5 in sterlet sperm and spots 3, 12, 14-16 in carp were phosphorylated on tyrosine during motility period. Incubation with anti-phosphoserine antibodies revealed an increased phosphorylation of spots 7, 12, 14–17 in sterlet spermatozoa and of spots 7-13 in carp after motility activation. Spot 5 in carp spermatozoa was dephosphorylated on serine residue after motility activation. Protein phosphorylation on threonine residue was weak in spermatozoa of both species. Only spot 2 in carp sperm was identified with anti-phosphothreonine antibodies.

Proteins of interest were cut from 2D gels (Fig. 11) and analysed with MALDI-TOF MS/MS. We were able to identify 18 of 38 selected protein spots in sterlet spermatozoa and 16 of 30 spots in carp. A detailed list of these sterlet and carp spermatozoa proteins involved in sperm motility is provided in Tables 2 and 3, respectively, together with their accession number, molecular mass, sequence coverage, number of unique peptides, and phosphorylated amino acid residue. The vast majority of identified proteins (14/18 for sterlet and 8/16 for carp) are involved in energy production, via oxidative phosphorylation or the glycolytic pathway. Among the others were cytoskeletal components, heat shock proteins, and calcium regulating enzymes. We discuss below the specificities of each of these findings and their potential meaning for fish sperm motility.
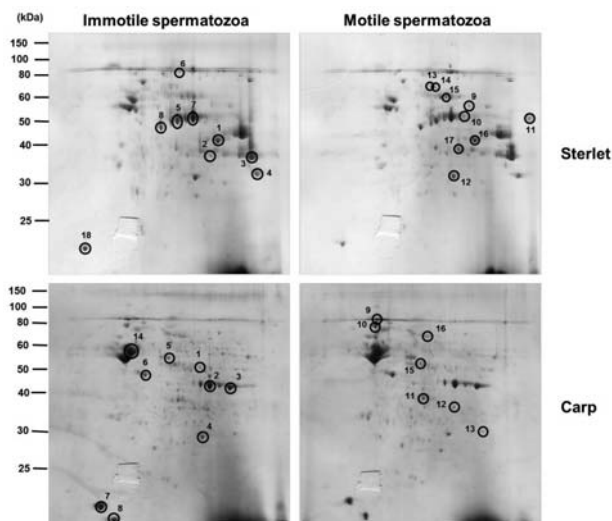
Figure 11. *Two-dimensional gel electrophoresis maps of sterlet and carp spermatozoa proteins. The separated proteins were stained with Coomassie Brilliant Blue R-250. Labelled spots correspond to proteins phosphorylated / dephosphorylated after motility activation.*

Table 2. Sterlet sperm protein identification by MALDI-TOF-MS/MS.

Phosphorylated amino acid	Protein Name [organism]	Accession no.	Calculated		MASCOT Score	Sequence Coverage (%)	Number of peptides (ion score ≥ 30)
			MW (kDa)	pI			
Spot 1 Serine/Threonine (PKA substrate)	PREDICTED: creatine kinase S-type, mitochondrial-like isoform X2 [*Neolamprologus brichardi*]	gi|584004697|ref|XP_006797349.1|	47.049	8.04	126	21	2
Spot 2 Serine/Threonine (PKA substrate)	Malate dehydrogenase, mitochondrial [*Danio rerio*]	gi|47085883|ref|NP_998296.1|	35.797	8.40	205	25	3
Spot 3 Serine/Threonine (PKA substrate)	Glyceraldehyde-3-phosphate dehydrogenase, partial [*Scaphirhynchus albus*]	gi|685015286|gb|AIN76876.1|	35.813	8.73	763	42	7
Spot 4 Serine/Threonine (PKA substrate)	PREDICTED: phosphoglycerate mutase 2-like [*Lepisosteus oculatus*]	gi|573874563|ref|XP_006625661.1|	28.925	8.64	265	38	4
Spot 5 Serine/Threonine (PKA substrate), Tyrosine	Phosphoglycerate kinase, partial [*Acipenser baerii*]	gi|46849425|dbj|BAD17922.1|	42.021	5.76	639	58	7
Spot 6 Serine (PKC substrate)	PREDICTED: dynein intermediate chain 2, ciliary-like [*Lepisosteus oculatus*]	gi|573876979|ref|XP_006626748.1|	79.01	5.56	315	11	3
Spot 7 Tyrosine, Serine	Enolase B [*Acipenser baerii*]	gi|98979421|gb|ABF60009.1|	47.515	7.60	1100	62	11
Spot 8 Tyrosine	PREDICTED: creatine kinase B-type [*Astyanax mexicanus*]	gi|597757318|ref|XP_007240392.1|	42.987	6.55	436	30	4

Table 2. *Continued.*

Spot	Protein	Accession					
Spot 9 Tyrosine	ATP synthase subunit alpha, mitochondrial [*Salmo salar*]	gi|213512628|ref|NP_011133132.1|	57.212	9.06	254	20	3
Spot 10 Tyrosine	PREDICTED: flotillin-1-like [*Oryzias latipes*]	gi|432883664|ref|XP_004074318.1|	48.030	6.32	101	23	1
Spot 11 Tyrosine	Septin-7-like isoform X3 [*Stegastes partitus*]	gi|657584138|ref|XP_008296208.1|	49.573	8.68	140	20	2
Spot 12 Tyrosine, Serine	Triosephosphate isomerase [*Acipenser brevirostrum*]	gi|15149246|gb|AAK85201.1|AF387818_1	27.126	6.32	401	67	5
Spot 13 Tyrosine	PREDICTED: phosphoglucomutase-1-like isoform X2 [*Lepisosteus oculatus*]	gi|573894328|ref|XP_006634914.1|	61.970	5.94	523	22	5
Spot 14 Serine	PREDICTED: phosphoglucomutase-1-like isoform X1 [*Lepisosteus oculatus*]	gi|573894326|ref|XP_006634913.1|	62.515	5.94	501	16	5
Spot 15 Serine	PREDICTED: pyruvate kinase PKM-like isoform X2 [*Lepisosteus oculatus*]	gi|573881641|ref|XP_006629014.1|	58.556	6.18	264	21	3
Spot 16 Serine	Creatine kinase [*Takifugu rubripes*]	gi|8575804	46.852	8.73	249	13	3
Spot 17 Serine	PREDICTED: L-lactate dehydrogenase A chain-like [*Lepisosteus oculatus*]	gi|573909562|ref|XP_006642499.1|	36.934	7.12	145	10	3
Spot 18 Tyrosine	Full=Calmodulin; Short=CaM [*Oreochromis mossambicus*]	gi|78099193|sp|Q6R520.3|CALM_OREMO	16.835	4.05	518	55	6

Table 3. Carp sperm protein identification by MALDI-TOF-MS/MS.

Phosphorylated amino acid	Protein Name [organism]	Accession no.	Calculated		MASCOT Score	Sequence Coverage (%)	Number of peptides (ion score ≥ 30)
			MW (kDa)	pI			
Spot 1 Serine/Threonine (PKA substrate)	PREDICTED: isocitrate dehydrogenase [NADP], mitochondrial-like [Poecilia formosa]	gi|617389834|ref|XP_007549128.1|	51.485	7.53	226	14	3
Spot 2 Serine/Threonine (PKA substrate), Threonine	PREDICTED: creatine kinase B-type isoform X1 [Poecilia reticulata]	gi|658914147|ref|XP_008396965.1|	43.718	6.9	368	21	3
Spot 3 Serine/Threonine (PKA substrate), Tyrosine	Brain creatine kinase [Danio rerio]	gi|116004537|ref|NP_001070631.1|	43.124	7.68	257	18	3
Spot 4 Serine/Threonine (PKA substrate), Tyrosine	Adenylate kinase isoenzyme 1 [Danio rerio]	gi|51571925|ref|NP_001003993.1|	21.485	7.68	381	34	5
Spot 5 Serine	Full=Septin-8-A [Danio rerio]	gi|223635831|sp|Q642H3.2|	49.253	5.9	389	28	5
Spot 6 Serine	Creatine kinase B-type [Ictalurus punctatus]	gi|576892111|gb|AHH42072.1|	43.079	5.3	567	20	5
Spot 7 Serine	Parvalbumin-7-like [Cynoglossus semilaevis]	gi|657802634|ref|XP_008328383.1|	12.134	5.26	129	33	2
Spot 8 Serine	Parvalbumin 6 [Danio rerio]	gi|45387575|ref|NP_991136.1|	12.034	4.93	275	27	2

Table 3. *Continued.*

Spot 9 Serine	PREDICTED: heat shock protein HSP 90-beta-like isoform X3 [*Lepisosteus oculatus*]	gi|573875316|ref|XP_006625988.1|	78.521	4.93	248	20	3
Spot 10 Serine	78 kDa glucose-regulated protein precursor (Heat shock 70 kDa protein 5) [*Danio rerio*]	gi|47085775|ref|NP_998223.1|	72.101	5.04	509	23	5
Spot 11 Serine	RecName: Full=L-lactate dehydrogenase B-A chain [*Danio rerio*]	gi|313104308|sp|Q9PVK4.4|	36.395	6.4	115	4	1
Spot 12 Serine, tyrosine	PREDICTED: malate dehydrogenase, mitochondrial-like [*Oryzias latipes*]	gi|432908348|ref|XP_004077823.1|	35.808	8.07	374	17	4
Spot 13 Serine	PREDICTED: parkin coregulated gene protein homolog [*Lepisosteus oculatus*]	gi|573887140|ref|XP_006631351.1|	27.225	7.72	171	18	3
Spot 14 Tyrosine	PREDICTED: tubulin alpha-1A chain-like, partial [*Astyanax mexicanus*]	gi|597760477|ref|XP_007244242.1|	44.182	5.01	704	34	5
Spot 15 Tyrosine	PREDICTED: flotillin-1 [*Poecilia formosa*]	gi|617438772|ref|XP_007564344.1|	47.739	6.71	124	10	1
Spot 16 Tyrosine	Transketolase-like protein 2 [*Danio rerio*]	gi|186288308|ref|NP_932336.3|	68.651	6.81	239	16	3

DISCUSSION

After spawning into external aqueous environment fish spermatozoa exhibit dramatic changes, from initiation of motility to acrosome reaction in some species. These changes are triggered by changes in the extracellular ionic environment and osmolality. Moreover, under natural conditions, a hypoosmotic environment, essential for spermatozoa activation of fresh water fish species, also induces osmotic and oxidative stress in sperm cells. After reception of extracellular signals by specific ion channels or receptors in the spermatozoa, intracellular signals are transferred to the axoneme through protein phosphorylation, Ca^{2+} signaling, and cAMP-dependent pathways (Inaba, 2003). However, for such evolutionarily distant fish species as common carp (*Cyprinus carpio* L.) and sturgeon (*Acipenser ruthenus*) not only does the trigger of motility activation differ, but the intracellular signaling pathways vary as well. Therefore the present study was undertaken in order to clarify which phospho-proteins and protein kinases are involved in sperm motility and intracellular signaling in these two important aquaculture species.

Protein kinases involved in sperm motility

Results of motility analyses in the presence of kinase inhibitors showed that PKA and PKC participate in motility activation in both species. Differences in susceptibility of sterlet and carp spermatozoa to the same concentrations of inhibitors could be due to differences in membrane permeability, as water transport across the membrane and volume changes during motility period vary greatly between these two species (Bondarenko et al., 2013). Also it should be mentioned that the specificity of inhibitors is never perfect, and various isoforms of kinases exist (White et al., 2007). Thus different types of kinase inhibitors may have stronger effects on spermatozoa motility of different species. Even though in the present study an inhibitor of protein tyrosine kinase (tyrphostin 23) did not show any significant effect on spermatozoa motility (Table 1), tyrosine phosphorylation of numerous sperm proteins was observed (Fig. 2), indicating that they are involved in sperm motility activation and regulation. Hence further studies with a series of PTK inhibitors are needed for further evaluation of the role of this kinase in fish spermatozoa motility.

Numerous studies have described the role of PKA in sperm motility of different species (Inaba, 2003; Lasko et al., 2012; Visconti et al., 1995). The activity of PKA depends on the amount of cAMP, which is in turn regulated by adenylate cyclase and phosphodiesterase. The binding of cAMP to the regulatory subunit of PKA promotes the dissociation and activation of the catalytic subunits that catalyse phosphorylation on serine/threonine residues (Urner and Sakkas, 2003). In fish spermatozoa involvement of PKA in motility was shown only for salmonids (Itoh et al., 2003), though cAMP-dependent regulation pathways were also reported in spermatozoa of *Sparus aurata* (Zilli et al., 2008) and *Polyodon spathula* (Linhart et al., 2002). In the current study results of inhibitory analysis, western blotting and immunolabeling with phospho-(Ser/Thr) PKA substrate antibody demonstrated that PKA is involved in motility activation and/or regulation in spermatozoa of *C. carpio* and *A. ruthenus*. This result was expected for sterlet, since sturgeon spermatozoa motility activation is cAMP-dependent (Linhart et al., 2002); however, carp spermatozoa motility activation was shown to be cAMP-independent (Cosson, Gagnon, 1988; Krasznai et al., 2000). Therefore it can be assumed that in carp spermatozoa, PKA activity is not involved directly in motility activation, but may participate in sustaining motility and its regulation.

Localization of PKA substrates and results of protein identification support this hypothesis (Fig. 9). Thus in both quiescent and motile sterlet spermatozoa PKA substrates were localized equally in the head and flagella. In contrast, results of immunolabeling of carp spermatozoa

showed only weak phosphorylation of PKA substrates in the midpiece of immotile spermatozoa, which was followed by increased phosphorylation of the midpiece and flagella after motility activation. It can be suggested that in quiescent carp spermatozoa, PKA is anchored in the midpiece and shows low activity in mitochondria; however after motility activation, activity of PKA increases in both mitochondria and cytosol. These results correlate with the results of protein identification by mass spectrometry, which showed that in carp spermatozoa PKA substrates phosphorylated after motility activation include mitochondrial protein isocitrate dehydrogenase [NADP] and cytoplasmic proteins creatine kinase and adenylate kinase (Table 2). All these enzymes are involved in ATP metabolism and play important roles in cellular energy homeostasis. Similar results were obtained for spermatozoa of sterlet, where identified PKA substrates involved in sperm motility were mitochondrial proteins: creatine kinase S-type and malate dehydrogenase; and cytoplasmic proteins: phosphoglycerate kinase, glyceraldehyde-3-phosphate dehydrogenase, and phosphoglycerate mutase. The obtained results suggest that in fish spermatozoa PKA is involved not only in phosphorylation of axonemal proteins as shown before (Itoh et al., 2003), but also plays an important role in oxidative phosphorylation and glycolytic pathway. Hence it is possible to speculate that PKA activity is necessary for ATP generation and/or transport along the flagella in order to sustain spermatozoa motility in carp and sterlet.

The protein kinase C (PKC) was shown to transduce signals during initiation and maintenance of motility in intact sea urchin spermatozoa (White et al., 2007) and in glycerol-treated salmonid sperm (Takei et al., 2012). Conventional PKC isoforms have a requirement for Ca^{2+} for their activation (Newton, 1995). Thus activity of PKC is strongly associated with and controlled by Ca^{2+} signaling. In the current study it was shown that in both species, *C. carpio* and *A. ruthenus*, sperm motility was blocked in presence of PKC inhibitor (chelerythrine) and results of western blotting and immunolabeling indicated presence of phosphorylated PKC substrates in spermatozoa of both species. Though we were not able to identify PKC substrate in carp spermatozoa due to low signal from antibodies, the PKC substrate in sterlet spermatozoa was identified as dynein intermediate chain 2. This result is in agreement with previous study on sea urchin spermatozoa which showed that the majority of the phospho-PKC substrate proteins, and PKC itself, were associated with the flagellum and axoneme (White et al., 2007).

Previous studies reported the involvement of PKC in acrosome reaction and gamete fusion (reviewed by Breitbart and Naor, 1999); however, less is known about the function of PKC in sperm motility activation. In human spermatozoa PKC was shown to localize in the head and in patches along the flagella, and to participate in sperm motility activation (Kalina et al., 1995). On the other hand studies on boar spermatozoa did not reveal any involvement of PKC in motility activation (Holt and Harrison, 2002), which is not surprising since boar spermatozoa do not require Ca^{2+} for motility activation (Tajima et al., 1987). In our studies the same concentrations of chelerythrine induced a more prominent effect on spermatozoa motility in sterlet than in carp, which correlates with results of western blotting and immunolabeling where signal provided by antibody to phospho-(Ser) PKC substrates in sterlet spermatozoa was stronger than in carp spermatozoa. Previous studies have shown that sturgeon spermatozoa motility activation is Ca^{2+}-dependent and addition of extracellular Ca^{2+} is able to reverse K^{+} inhibitory effect (Alavi et al., 2011). In contrast, spermatozoa of another cyprinid fish (*Puntius javanicus*) were shown to be activated in the absence of Ca^{2+} and addition of external Ca^{2+} inhibited motility (Morita et al., 2006a). Overall these results may suggest that involvement of Ca^{2+}-dependent PKC in spermatozoa motility is species-specific and reflects the ability of Ca^{2+} to stimulate motility.

Phospho-proteins involved in sperm motility

We observed that many proteins changed their phosphorylation status after motility activation (Figs. 1–3). Unfortunately we were not able to identify all phospho-proteins in carp and sterlet spermatozoa due to the absence of protein databases and incomplete genome sequencing. Nevertheless we identified 6 serine-phosphorylated proteins involved in sterlet spermatozoa motility: phosphoglucomutase-1-like, pyruvate kinase PKM-like, creatine kinase, L-lactate dehydrogenase A chain, triose phosphate isomerase, enolase B. All these enzymes participate in glycolytic pathway and regulate energy production. Results of immuno-fluorescence labeling indicate that phospho-serine proteins are localized in the head and along the flagellum of sterlet spermatozoon. Increased intensity of fluorescence after motility activation correlate with results of western blotting and may reflect activation of glycolytic processes. The abundance of metabolic enzymes participating in sterlet sperm motility may explain the relatively long period of their movement (up to 3 min), compared to other freshwater fish species (60 sec in carp), as well as slow ATP decrease in spermatozoa during motility period (Billard et al., 1999).

In contrast, among serine-phosphorylated carp spermatozoa proteins only creatine kinase B-type, L-lactate dehydrogenase B-A chain, and malate dehydrogenase belong to energy metabolic pathways. The rest of the serine phospho-proteins are axonemal and cytosolic proteins: septin-8-A, parvalbumin-7-like, parvalbumin 6, heat shock protein HSP 90, 78 kDa glucose-regulated protein precursor (heat shock 70kDa protein 5), and parkin coregulated gene protein homolog.

It should be mentioned that septin-8-A is a member of the highly conserved septin family of GTP-binding proteins that co-localizes with cellular membranes and the microtubule cytoskeleton (Spiliotis, 2010). In our previous study we found that septin-8-A is dephosphorylated under oxidative stress in carp spermatozoa (Gazo et al., 2015), and a similar result was obtained in the current study after sperm motility activation in hypotonic conditions. Also previous studies reported that septin-8 is a substrate for MAPK-activated protein kinase 5 (MK5) (Shiryaev et al., 2012). MAPKs are key regulatory enzymes in cell signaling, participating in diverse cellular functions and consist of several tiers of protein kinases that activate each other by sequential phosphorylation. Furthermore, heat shock proteins are known to be phosphorylated by MAPK-activated protein kinase under osmotic stress (Rouse et al., 1994). In the current study two heat shock proteins were phosphorylated in carp spermatozoa after motility activation. Interestingly, it was found that MAPK cascade functions in parallel with the Ca^{2+} signaling during sperm activation in *Caenorhabditis elegans*, and activation of the MAPK cascade bypasses the requirement of Ca^{2+} to induce sperm activation (Liu et al., 2014). Overall, these data make MAPK cascade a promising candidate for spermatozoa motility regulation in common carp which possess the osmotic mode of sperm motility activation.

Heat shock proteins (HSPs) are highly conserved, critical components of the cell stress response. HSPs are classified into families on the basis of their molecular weight, and they are overexpressed during acute and chronic stress. The role of HSPs in spermatozoa has been studied in mammals (Cole and Meyers, 2011) and ascidians (Satouh et al., 2005). HSPs are classified as chaperone proteins, which protect intracellular macromolecules against unfolding and aggregation. Heat shock protein 70 (HSP 70) and heat shock protein 90 (HSP 90) are present in the cytosol and nucleus of somatic cells; they refold and transport proteins, and play an important role in the cellular distribution of proteins (Kakar et al, 2006). Previous studies also reported an increase in the phosphorylation of HSP 70 under osmotic stress (Cole and Meyers, 2011), and of HSP 90 during human sperm capacitation (Li et al., 2014). Moreover, HSP 70 is known to have a protective effect on cells undergoing heat or osmotic stress (Huang et al., 2000; Cole and Meyers, 2011). HSP 70 was shown to localize in the head and HSP 90 in the midpiece of monkey spermatozoa (Cole and Meyers, 2011). This

corresponds to our results of immunolabeling, which showed an increased phosphorylation on serine residues in the head and midpiece of carp spermatozoa after motility activation. Overall these data indicate involvement of HSP 90 and HSP 70 in carp sperm motility activation under hypotonic conditions.

Parvalbumin (PV), phosphorylated upon motility activation in spermatozoa of common carp, is a calcium-binding protein with three EF-hand motifs, structurally related to calmodulin (CaM). A previous study reported the presence of PV in carp spermatozoa (Dietrich et al., 2010); however, results of the current study demonstrated its participation in sperm motility and indicated that PV function is controlled by phosphorylation. Interestingly, compared to other Ca^{2+}-binding proteins, such as CaM, PV has a slow dissociation and association rate, due to the fact that Mg^{2+} ions compete with Ca^{2+} ions for binding (Caillard et al., 2000). Hence it is possible that the presence of PV in carp spermatozoa suggests a different Ca^{2+} signaling mechanism, compared to spermatozoa which contain CaM such as sterlet (Table 2) or tilapia (Morita et al., 2006b). Further studies are needed to understand the role of PV in spermatozoa of common carp.

In both species some of the tyrosine-phosphorylated proteins belong to ATP production and energy homeostasis pathways: malate dehydrogenase, transketolase, adenylate kinase and creatine kinase in carp spermatozoa; enolase B, phosphoglycerate kinase, creatine kinase B-type, ATP synthase subunit alpha, triosephosphate isomerase and phosphoglucomutase-1-like isoform X2 in sterlet spermatozoa. Similar to phosphorylation on serine residue, the most abundant tyrosine phospho-proteins in sterlet spermatozoa were proteins associated with glycolysis and energy metabolism. Results of western blotting and immunolabeling indicate increased tyrosine phosphorylation right after motility activation and slow dephosphorylation of tyrosine residue in proteins up to the end of motility period.

Furthermore, we found out that CaM and septin-7 are also involved in sterlet sperm motility and regulated by tyrosine phosphorylation. Involvement of CaM in sperm motility has been reported for many other aquatic species, such as tilapia (Morita et al., 2006b) and ascidians (Nomura et al., 2004). CaM is considered to be a ubiquitous protein mediating intracellular Ca^{2+} signaling which activates sperm motility through the activation of dynein ATPase (Tash et al., 1988). Hence obtained results are in agreement with previous studies, which showed Ca^{2+}-dependence of sturgeon sperm motility activation (Alavi et al., 2011).

Studies on mammals showed that septins are localized in the annulus – a fibrous structure between midpiece and principal piece of the sperm tail. Also, partial co-localization with microtubules has been reported for septin 7 (Spiliotis, 2010). The role of septins in fish sperm motility is not clear yet. However, it can be suggested that microtubule-associated septins may spatially modulate the association of tubulin with post-translationally modifying enzymes and microtubule motors.

This assumption is supported by increased alpha-tubulin phosphorylation in carp spermatozoa, where septin-8-A was also dephosphorylated after motility activation. Tubulin belongs to a highly conserved multigenic family, in which several gene products usually coexist in the same cell (Mohri et al., 2012). Non-receptor tyrosine kinases such as pp60c-src, Fes, Jak2, and Syk phosphorylate a tyrosine residue(s) on tubulin (Wandosell et al., 1987). Immunofluorescent localization of phospho-tyrosine in the current study indicates increased phosphorylation in flagella of carp spermatozoa after motility activation. This result is in agreement with tubulin localization shown by Tash and Means (1982) in dog sperm. The

physiological relevance of alpha-tubulin phosphorylation in fish spermatozoa remains to be determined. However, previous studies showed that phosphorylation of alpha-tubulin on tyrosine residues can impair tubulin polymerization *in vitro* (Wandosell et al., 1987).

Flotillin-1 was identified in spermatozoa of both species and was phosphorylated on tyrosine residue during motility period. Flotillin-1 is a membrane-associated protein which is thought to function in a number of cellular contexts, including signaling, endocytosis and interactions with the cytoskeleton. It has been shown that capacitation of boar spermatozoa induced redistribution of flotillin-1 in plasma membrane of the spermatozoon head (van Gestel et al., 2005). Moreover, tyrosine phosphorylation is known to control distribution of flotillin microdomains (Riento et al., 2009). In our study tyrosine phosphorylation of the head region of both carp and sterlet spermatozoa increased after motility initiation, which may be an indicator of the reorganization of lipid rafts after motility initiation.

Results of western blotting and immuno-fluorescence labeling also indicated that protein phosphorylation on threonine residue is involved in sperm motility activation (Figs. 3, 8). However, the only threonine-phosphorylated protein we were able to identify is creatine kinase B-type isoform X1 in carp spermatozoa. This protein was also shown to be a phospho-(Ser/Thr) PKA substrate. Immunolocalization of phospho-threonine in carp and sterlet spermatozoa showed that the majority of proteins phosphorylated on threonine residue were localized in the midpiece of spermatozoa. These results indicate a partial overlapping of PKA substrates and phospho-threonine proteins.

CONCLUSION

In conclusion, sperm protein phosphorylation patterns differ significantly between two species, *C. carpio* L. and *A. ruthenus*. Differences in the way motility activation is triggered are followed by variations in intracellular signaling and implementation of secondary messengers. Though PKA and PKC are important participants in sperm motility, the roles of these two kinases seem to be different between two species. Thus PKA seems to play a role only in energy production and sustaining of carp spermatozoa motility. However, it has been shown that carp spermatozoa movement mainly depends on ATP stored before activation (Perchec et al., 1995). Therefore cAMP signaling is not a prerequisite of successful motility activation in *C. carpio*. On the other hand, the abundance of glycolytic enzymes in sterlet spermatozoa may indicate dependence of sperm movement on glycolytic ATP production during motility period and cAMP-dependent PKA signaling.

Furthermore, the difference in phospho-(Ser) PKC substrate abundance between carp and sterlet spermatozoa, as well as presence of PV instead of CaM in carp sperm, may reflect different involvement of Ca^{2+} signaling in sperm motility. Further studies are needed to reveal mechanisms of Ca^{2+} signaling during sperm motility activation in these two species.

An increased intensity of serine and tyrosine phosphorylation in the head of carp and sterlet spermatozoa may reflect an activation of signaling pathways involved in gamete fusion and fertilization, considering that sterlet spermatozoon possesses a functional acrosome, in contrast to carp sperm which is devoid of acrosome.

Involvement of multiple phospho-proteins in spermatozoa motility of carp and sterlet indicates a complex network of signaling molecules and enzymes involved in sperm motility activation, regulation and fertilization. This work has set the stage for future studies by identifying the major phospho-proteins whose functions should be analyzed in detail.

Molecular mechanisms of motility activation and intracellular signaling in spermatozoa of two taxonomically distant fish species: sterlet (Acipenser ruthenus) and common carp (Cyprinus carpio L.)

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REFERENCES

Aitken, R.J., Paterson, M., Fisher, H., Buckingham, D.W., van Duin, M., 1995. Redox regulation of tyrosine phosphorylation in human spermatozoa and its role in the control of human sperm function. J. Cell Sci. 108, 2017–2025.

Alavi, S.M.H., Cosson, J., 2006. Sperm motility in fishes. (II) Effects of ions and osmolality: A review. Cell Biol. Int. 30, 1–14.

Alavi, S.M.H., Gela, D., Rodina, M., Linhart, O., 2011. Roles of osmolality, calcium — Potassium antagonist and calcium in activation and flagellar beating pattern of sturgeon sperm. Comp. Biochem. Physiol. A 160, 166–174.

Billard, R., Cosson, J., Fierville, F., Brun, R., Rouault, T., Williot, P., 1999. Motility analysis and energetics of the Siberian sturgeon *Acipenser baerii* spermatozoa. J. Appl. Ichthyol. 15, 199–203.

Bondarenko, O., Dzyuba, B., Cosson, J., Yamaner, G., Prokopchuk, G., Psenicka, M., Linhart, O., 2013. Volume changes during the motility period of fish spermatozoa: interspecies differences. Theriogenology 79, 872–881.

Breitbart, H., Naor, Z., 1999. Protein kinases in mammalian sperm capacitation and the acrosome reaction. Rev. Reprod. 4, 151–159.

Brokaw, C.J., 1987. A lithium-sensitive regulator of sperm flagellar oscillation is activated by cAMP-dependent phosphorylation. J. Cell Biol. 105, 1789–1798.

Caillard, O., Moreno, H., Schwaller, B., Llano, I., Celio, M.R., Marty, A., 2000. Role of the calcium-binding protein parvalbumin in short-term synaptic plasticity. Proc. Natl. Acad. Sci. U S A. 97, 13372–13377.

Cole, J.A., Meyers, S.A., 2011. Osmotic stress stimulates phosphorylation and cellular expression of heat shock proteins in rhesus macaque sperm. J. Androl. 32, 402–410.

Cosson, M.-P., Gagnon, C., 1988. Protease inhibitors and substrates block motility and microtubule sliding of sea urchin and carp spermatoza. Cell. Motil. Cytoskeleton 10, 518–527.

Dietrich, M.A., Nynca, J., Bilińska, B., Kuba, J., Kotula-Balak, M., Karol, H., Ciereszko, A., 2010. Identification of parvalbumin-like protein as a major protein of common carp (*Cyprinus carpio* L.) spermatozoa which appears during final stage of spermatogenesis. Comp. Biochem. Physiol. B 157, 220–227.

Frenkerl, G., Peterson, R.N., Freund, M., 1973. The role of adenine nucleotides and the effect of caffeine and dibutyryl cyclic AMP on the metabolism of guinea pig epididymal spermatozoa. Proc. Soc. Exp. Biol. Med. 144, 420–425.

Gazo, I., Shaliutina-Kolesova, A., Dietrich, M.A., Linhartová, P., Shaliutina, O., Cosson, J. 2015. The effect of reactive oxygen species on motility parameters, DNA integrity, tyrosine phosphorylation and phosphatase activity of common carp (*Cyprinus carpio* L.) spermatozoa. Mol. Reprod. Dev. 82, 48–57.

Hamasaki, T., Barkalow, K., Richmond, J., Satir, P., 1991. cAMP-stimulated phosphorylation of an axonemal polypeptide that copurifies with the 22S dynein arm regulates microtubule translocation velocity and swimming speed in Paramecium. Proc. Natl. Acad. Sci. USA 88, 7918–7922.

Hayashi, H., Yamamoto, K., Yonekawa, H., Morisawa, M., 1987. Involvement of tyrosine protein kinase in the initiation of flagellar movement in rainbow trout spermatozoa. J. Biol. Chem. 262, 16692–16698.

Holt, W.V., Harrison, R.A.P., 2002. Bicarbonate stimulation of boar sperm motility via a protein kinase A–dependent pathway: between-cell and between-ejaculate differences are not due to deficiencies in protein kinase A activation. J. Androl. 23, 557–565.

Hopper, R.K., Carroll, S., Aponte, A.M., Johnson, D.T., French, S., Shen, R.-F., Witzmann, F.A., Harris, R.A., Balaban, R.S., 2006. Mitochondria matrix phosphoproteome: effect of extra mitochondrial calcium. Biochemistry 45, 2524–2536.

Huang, S.Y., Kuo, Y.H., Lee, Y.P., Tsou, H.L., Lin, E.C., Ju, C.C., Lee, W.C., 2000. Association of heat shock protein 70 with semen quality in boars. Anim. Reprod. Sci. 63, 231–240.

Huttemann, M., Lee, I., Samavati, L., Yu, H., Doan, J.W., 2007. Regulation of mitochondrial oxidative phosphorylation through cell signaling. Biochim. Biophys. Acta 1773, 1701–1720.

Inaba, K., Morisawa, S., Morisawa M., 1998. Proteasomes regulate the motility of salmonid fish sperm through modulation of cAMP-dependent phosphorylation of an outer arm dynein light chain. J. Cell Sci. 111, 1105–1115.

Inaba, K., 2003. Molecular architecture of the sperm flagella: Molecules for motility and signaling. Zoolog. Sci. 20, 1043–1056.

Inaba, K., 2007. Molecular basis of sperm flagellar axonemes: structural and evolutionary aspects. Ann. N. Y. Acad. Sci. 1101, 506–526.

Inaba, K., Kagami, O., Ogawa, K., 1999. Tctex2-related outer arm dynein light chain is phosphorylated at activation of sperm motility. Biochem. Biophys. Res. Commun. 256, 177–183.

Itoh, A., Inaba, K., Ohtake, H., Fujinoki, M., Morisawa, M., 2003. Characterization of a cAMP-dependent protein kinase catalytic subunit from rainbow trout spermatozoa. Biochem. Biophys. Res. Commun. 305, 855–861.

Kakar, M., Kanwal, C., Davis, J.R., Li, H., Lim, C.S., 2006. Geldanamycin, an inhibitor of Hsp90, blocks cytoplasmic retention of progesterone receptors and glucocorticoid receptors via their respective ligand binding domains. AAPS J. 8, E718–E728.

Kalina, M., Socher, R., Rotem, R., Naor, Z., 1995. Ultrastructural localization of protein kinase C in human sperm. J. Histochem. Cytochem. 43, 439–445.

Krasznai, Z., Marian, T., Izumi, H., Damjanovich, S., Balkay, L., Tron, L., Morisawa, M., 2000. Membrane hyperpolarization removes inactivation of Ca^{2+} channels, leading to Ca^{2+} influx and subsequent initiation of sperm motility in the common carp. Proc. Natl. Acad. Sci. USA 97, 2052–2057.

Krisfalusi, M., Miki, K., Magyar, P.L., O'Brien, D.A., 2006. Multiple glycolytic enzymes are tightly bound to the fibrous sheath of mouse spermatozoa. Biol. Reprod. 75, 270–278.

Lasko, J., Schlingmann, K., Klocke, A., Mengel, G.A., Turner, R., 2012. Calcium/calmodulin and cAMP/protein kinase-A pathways regulate sperm motility in the stallion. Anim. Reprod. Sci. 132, 169–177.

Li, K., Xue, Y., Chen, A., Jiang, Y., Xie, H., Shi, Q., Zhang, S., Ni, Y., 2014. Heat shock protein 90 has roles in intracellular calcium homeostasis, protein tyrosine phosphorylation regulation, and progesterone-responsive sperm function in human sperm. PLoS One 9, e115841.

Lindemann, C.B., Kanous, K.S., 1989. Regulation of mammalian sperm motility. Arch. Androl. 23, 1–22.

Linhart, O., Cosson, J., Mims, S.D., Shelton, W. L., Rodina, M., 2002. Effects of ions on the motility of fresh and demembranated paddlefish (*Polyodon spathula*) spermatozoa. Reproduction 124, 713–719.

Liu, Z., Wang, B., He, R., Zhao, Y., Miao, L., 2014. Calcium signaling and the MAPK cascade are required for sperm activation in *Caenorhabditis elegans*. Biochim. Biophys. Acta 1843, 299–308.

Mansour, N., Lahnsteiner, F., Berger, B., 2003. Metabolism of intratesticular spermatozoa of a tropical teleost fish (*Clarias gariepinus*). Comp. Biochem. Physiol. B 135, 285–296.

Mohri, H., Inaba, K., Ishijima, S., Baba, S.A., 2012. Tubulin-dynein system in flagellar and ciliary movement. Proc. Jpn. Acad., Ser. B 88, 397–415.

Morita, M., Takemura, A., Okuno, M., 2003. Requirement of Ca^{2+} on activation of sperm motility in euryhaline tilapia *Oreochromis mossambicus*. J. Exp. Biol. 206, 913–921.

Morita, M., Okuno, M., Susilo, E.S., Setyo, B.P., Martarini, D., Harnadi, L., Takemura, A., 2006a. Changes in sperm motility in response to osmolality/Ca^{2+} in three Indonesian fresh water teleosts: goby (*Oxyeleotris marmorata*), Java carp (*Puntius javanicus*), and catfish (*Clarias batrachus*). Comp. Biochem. Physiol. A Mol. Integr. Physiol. 143, 361–367.

Morita, M., Takemura, A., Nakajima, A., Okuno, M., 2006b. Microtubule sliding movement in tilapia sperm flagella axoneme is regulated by Ca^{2+}/calmodulin-dependent protein phosphorylation. Cell Motil. Cytoskeleton 63, 459–470.

Nascimento, J.M., Shi, L.Z., Tam, J., Chandsawangbhuwana, C., Durrant, B., Botvinick, E.L., Berns, M.W., 2008. Comparison of glycolysis and oxidative phosphorylation as energy sources for mammalian sperm motility, using the combination of fluorescence imaging, laser tweezers, and real-time automated tracking and trapping. J. Cell. Physiol. 217, 745–751.

Newton, A.C., 1995. Protein kinase C: structure, sunction, and regulation. J. Biol. Chem. 270, 28495–28498.

Nomura, M., Inaba, K., Morisawa, M., 2000. Cyclic AMP- and calmodulin-dependent phosphorylation of 21 and 26 kda proteins in axoneme is a prerequisite for SAAF-induced motile activation in ascidian spermatozoa. Dev. Growth Differ. 42, 129–138.

Nomura, M., Yoshida, M., Morisawa, M., 2004. Calmodulin/calmodulin-dependent protein kinase II mediates SAAF-induced motility activation of ascidian sperm. Cell Motil. Cytoskeleton 59, 28–37.

O'Flaherty, C., de Lamirande, E., Gagnon, C., 2006. Reactive oxygen species modulate independent protein phosphorylation pathways during human sperm capacitation. Free Radic. Biol. Med. 40, 1045–1055.

Pagliarini, D.J., Dixon, J.E., 2006. Mitochondrial modulation: reversible phosphorylation takes center stage? Trends Biochem. Sci. 31, 26–34.

Perchec, G., Jeulin, C., Cosson, J., André, F., Billard, R., 1995. Relationship between sperm ATP content and motility of carp spermatozoa. J. Cell Sci. 108, 747–753.

Perchec Poupard, G., Gatti, J.L., Cosson, J., Jeulin, C., Fierville, F., Billard, R., 1997. Effects of extracellular environment on the osmotic signal transduction involved in activation of motility of carp spermatozoa. J. Reprod. Fertil. 110, 315–327.

Psenicka, M., Alavi, S. M. H., Rodina, M., Gela, D., Nebesarova, J., Linhart, O., 2007. Morphology and ultrastructure of Siberian sturgeon *Acipenser baerii* spermatozoa using scanning and transmission electron microscopy. Biol. Cell 99, 103–115.

Ray, P.D., Huang, B.W., Tsuji Y., 2012. Reactive oxygen species (ROS) homeostasis and redox regulation in cellular signaling. Cell Signal. 24, 981–990.

Riento, K., Frick, M., Schafer, I., Nichols, B.J., 2009. Endocytosis of flotillin-1 and flotillin-2 is regulated by Fyn kinase. J. Cell Sci. 122, 912–918.

Rodina, M., Gela, D., Kocour, M., Alavi, S.M.H., Hulak, M., Linhart, O., 2007. Cryopreservation of tench, *Tinca tinca*, sperm: sperm motility and hatching success of embryos. Theriogenology 67, 931–940.

Rouse, J., Cohen, P., Trigon, S., Morange, M., Alonso-Llamazares, A., Zamanillo, D., Hunt, T., Nebreda, A.R., 1994. A novel kinase cascade triggered by stress and heat shock that stimulates MAPKAP kinase-2 and phosphorylation of the small heat shock proteins. Cell 78, 1027–1037.

Satouh, Y., Padma, P., Toda, T., Satoh, N., Ide, H., Inaba, K., 2005. Molecular characterization of radial spoke subcomplex containing radial spoke protein 3 and heat shock protein 40 in sperm flagella of the ascidian *Ciona intestinalis*. Mol. Biol. Cell 16, 626–636.

Shiryaev, A., Kostenko, S., Dumitriu, G., Moens, U., 2012. Septin 8 is an interaction partner and *in vitro* substrate of MK5. World J. Biol. Chem. 3, 98–109.

Spiliotis, E.T., 2010. Regulation of microtubule organization and functions by septin GTPases. Cytoskeleton (Hoboken) 67, 339–345.

Storey, B.T., 2008. Mammalian sperm metabolism: oxygen and sugar, friend and foe. Int. J. Dev. Biol. 52, 427–437.

Tajima, Y., Okamura, N., Sugita, Y., 1987. The activating effects of bicarbonate on sperm motility and respiration at ejaculation. Biochim. Biophys. Acta. 924, 519–529.

Takei, G.L., Mukai, C., Okuno, M., 2012. Transient Ca^{2+} mobilization caused by osmotic shock initiates salmonid fish sperm motility. J. Exp. Biol. 215, 630–641.

Tash, J.S., Krinks, M., Patel, J., Means, R.L., Klee, C.B., Means, A.R., 1988. Identification, characterization, and functional correlation of calmodulin-dependent protein phosphatase in sperm. J. Cell Biol. 106, 1625–1633.

Tash, J.S., Means, A.R., 1982. Regulation of protein phosphorylation and motility of sperm by cyclic adenosine monophosphate and calcium. Biol. Reprod. 26, 745–763.

Urner, F., Sakkas, D., 2003. Protein phosphorylation in mammalian spermatozoa. Reproduction 125, 17–26.

van Gestel, R.A., Brewis, I.A., Ashton, P.R., Helms, J.B., Brouwers, J.F., Gadella, B.M., 2005. Capacitation-dependent concentration of lipid rafts in the apical ridge head area of porcine sperm cells. Mol. Hum. Reprod. 11, 583–590.

Visconti, P.E., Moore, G.D., Bailey, J.L., Leclerc, P., Connors, S.A., Pan, D., Olds-Clarke, P., Kopf, G.S., 1995. Capacitation of mouse spermatozoa. II. Protein tyrosine phosphorylation and capacitation are regulated by a cAMP-dependent pathway. Development 121, 1139–1150.

Wandosell, F., Serrano, L., Avila, J., 1987. Phosphorylation of alpha-tubulin carboxyl-terminal tyrosine prevents its incorporation into microtubules. J. Biol. Chem. 262, 8268–8273.

White, D., de Lamirande, E., Gagnon, C., 2007. Protein kinase C is an important signaling mediator associated with motility of intact sea urchin spermatozoa. J. Exp. Biol. 210, 4053–4064.

Zilli, L., Schiavone, R., Storelli, C., Vilella, S., 2008. Molecular mechanisms determining sperm motility initiation in two sparids (*Sparus aurata* and *Lithognathus mormyrus*). Biol. Reprod. 79, 356–366.

CHAPTER 6

GENERAL DISCUSSION
ENGLISH SUMMARY
CZECH SUMMARY
ACKNOWLEDGMENTS
LIST OF PUBLICATIONS
TRAINING AND SUPERVISION PLAN DURING THE STUDY
CURRICULUM VITAE

GENERAL DISCUSSION

Though spermatozoa of externally fertilising species are particularly vulnerable to oxidative damage, only few studies have dealt with ROS production in fish spermatozoa (Hagedorn et al., 2012) and their effects, especially for ROS generated by water pollutants (Zhou et al., 2006). Moreover, reproduction of numerous fish species used in aquaculture depends on procedures of artificial fertilization. Therefore it is necessary not only to know how sperm cells are constructed, but also which mechanisms of stress protection they apply, and how spermatozoa motility is initiated and regulated in freshwater fish. The current study was undertaken in order to understand how production of ROS and subsequent oxidative stress influence spermatozoa of teleostean and chondrostean fish species, how the protein phosphorylation is involved in stress response and sperm functioning. Additionally, protective effect of seminal plasma and antioxidant enzymes on carp spermatozoa exposed to oxidative stress was investigated.

The first aim of the present study was to determine the effect of xenobiotic-induced oxidative stress on fish spermatozoa quality parameters after *in vitro* exposure. It has been shown that incubation of sterlet spermatozoa with xenobiotics *in vitro* led to decreased sperm motility and velocity (Chapter 2; Hulak et al., 2013; Linhartova et al., 2013). Sperm motility is a key prerequisite determining the quality and fertilizing ability of semen. Since variations in spermatozoa motility might reflect quantitative and qualitative toxic effects of ectogenous contaminants, monitoring of spermatozoa motility parameters is an important approach in toxicity experiments (Rurangwa et al., 2004). Decreased sperm motility represents one of the adverse effects of water pollutants, which can affect individual fertility and, hence, poses problems for the whole fish population. On the other hand, analysis of sperm motility allows detecting active doses that impairs sperm motility function but is not enough to give detailed explanations about mechanisms of xenobiotic toxicity. There are several possible ways by which EDCs may affect spermatozoa motility activation and movement. EDCs can act either through binding to membrane receptors and influencing intracellular signaling such as $[Ca^{2+}]_i$ or kinase activity, or through binding to nuclear receptors, provided its permeability through the cytoplasmic membrane. Nuclear receptors translocate into the nucleus upon binding of the xenobiotic to trigger a specific transcriptional response of the cell. As a result adverse developmental, reproductive, neurological, carcinogenic and immune effects can be produced in both humans and wildlife. However, it is recognized that spermatozoa are transcriptionally inactive cells. Therefore one of the possible mechanisms of xenobiotic effect is binding to membrane receptors. Despite the limited amount of information about membrane receptors in fish spermatozoa, it is noteworthy that Thomas and Doughty (2004) observed that non-estrogenic as well as estrogenic organic compounds could interfere with a rapid non-genomic progestin action to up-regulate spermatozoa motility in Atlantic croaker (*Micropogonias undulatus*). Further studies will be performed to elucidate the effect of EDCs on intracellular signaling in spermatozoa of sterlet.

Results obtained in the present study allow us to suggest that EDCs are able to affect fish spermatozoa through an increased ROS production. An inverse correlation has been detected between sperm motility and lipid / protein oxidation after exposure of spermatozoa to xenobiotics (Chapter 2; Hulak et al., 2013). Increased levels of LO and CP indicate that xenobiotics have induced oxidative stress in sterlet spermatozoa. This is in agreement with previous studies of Zhou et al. (2006), who showed a correlation between duroquinone concentration, decrease of spermatozoa motility, lipid oxidation and ROS production. Furthermore the xanthine-xanthine oxidase (X–XO) system is known to generate ROS that are involved in spermatozoa damage (Griveau et al., 1995). In our studies, incubation of

spermatozoa of common carp with X–XO leads to motility decrease, increased levels of CP and LO, and DNA damage (Chapter 3), all features similar to those observed in sterlet spermatozoa exposed to xenobiotics. However, the mechanism of EDC-induced ROS production in fish spermatozoa remains to be better clarified.

Lipid oxidation can trigger the loss of membrane integrity, causing increased cell permeability and enzyme inactivation (Aitken et al., 1993), which, in turn, can affect sperm motility. Violation of membrane integrity reduces the ability of spermatozoon to withstand osmotic shock occurring at motility activation. Thus increased levels of LO predict disruption of some spermatozoa function. It is known that, in addition to lipids, free radicals can also react with some protein residues. Protein carbonyl formation has been shown to be an early marker for protein oxidation (Shiva et al., 2011). 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 environmental concentrations of vinclozolin led to increased LO and CP levels in sterlet spermatozoa (Chapter 2), whereas similar increase of LO and CP was observed in spermatozoa of common carp incubated with X–XO (Chapter 3). Therefore, based on our results, we hypothesize that one possible way to explain motility parameters decline in fish spermatozoa *in vitro* could be the loss of membrane integrity as well as enzyme inactivation caused by oxidative stress.

Another possible explanation of ROS-mediated decline of sperm motility parameters is a decrease of ATP content. ATP is essential for spermatozoa motility, and decreased ATP equates with decreased spermatozoa movement (Perchec et al., 1995). It has been shown that after incubation with environmentally relevant concentrations of xenobiotics ATP content in sterlet spermatozoa decreased significantly (Chapter 2; Hulak et al., 2013; Linhartova et al., 2013). This could be due to ROS-mediated inhibition of one or more enzymes of oxidative phosphorylation and/or glycolysis, which limited the generation of ATP. In sterlet spermatozoa, numerous metabolic enzymes, including creatine kinase, were shown to participate in sperm motility activation and sustaining (Chapter 5). Studies on creatine kinase (CK) activity in myofibrils showed that CK can be inhibited by ROS, probably by the oxidation of its essential sulfhydryl groups (Mekhfi et al., 1996). Abundance of CK in spermatozoa of sterlet suggests its importance in energy production pathways. Moreover, participation of numerous metabolic enzymes in sterlet spermatozoa motility may explain the relatively long period of their movement, compared to other freshwater species (Chapter 5). Hence, suppression of CK activity by ROS could lead to decreased intracellular ATP content in spermatozoa exposed to xenobiotics and oxidative stress.

Studies of human spermatozoa have revealed that oxidative stress may induce significant DNA damage in both the mitochondrial and nuclear genomes (Aitken and Baker, 2006). Using the comet assay we were able to show that vinclozolin, as well as ROS produced by X–XO system, damage spermatozoa DNA in models of chondrostean and teleostean fish (Chapters 2 and 4). Moreover, both spermatozoa motility and DNA integrity were shown to inversely correlate with indicators of oxidative stress such as LO and CP (Chapter 2), thus DNA fragmentation constitutes a reliable evaluation of free-radical mediated damage induced by oxidative stress (Kodoma et al., 1997). DNA strand breaks induced by ROS are potential pre-mutagenic lesions and are sensitive markers of chemical genotoxicity (Morales et al., 2013). The damaged DNA from spermatozoa could be transmitted to the offspring, and cause the negative effects in development (decrease in hatching rate, higher level of larval abnormalities) and growth (Barranger et al., 2014). Hence obtained results allow us to hypothesize that high sensitivity of sterlet spermatozoa toward the presence of xenobiotics makes them a promising model for studying effects of water pollution on reproduction of

aquatic organisms. On the other hand current results also emphasize the threat of water pollution for wild population of sturgeons.

To counteract the damaging effects of ROS, a variety of antioxidant enzymes are naturally present in fish spermatozoa and seminal fluid (Li et al., 2010). Therefore, our second aim was to evaluate the protective properties of antioxidants against oxidative stress in spermatozoa of common carp. Superoxide dismutase is considered the first line of defence against the effects of free radicals in the cell through catalysing the dismutation of superoxide radicals to hydrogen peroxide and molecular oxygen. In the present study, significantly increased SOD activity was observed in spermatozoa exposed to xenobiotic and ROS (Chapters 2 and 3). Furthermore, carp spermatozoa exposed to X–XO also showed an increase of glutathione reductase and glutathione peroxidase activity in a time- and dose-dependent manner. This is likely to be an adaptive response to toxicant stress and serves to neutralize the impact of increased ROS generation. Spermatozoa have limited antioxidant capacity to defend against ROS attack and are highly dependent on their intrinsic intracellular antioxidants and the antioxidant protection of the seminal plasma (Koziorowska-Gilun et al., 2011). Therefore it is not surprising that the addition of seminal plasma and antioxidants to spermatozoa samples exposed to the X–XO system significantly improved spermatozoa performance (Chapter 3), suggesting reduction in the damaging effects of ROS.

It is well known that ROS are able to modulate spermatozoa intracellular signaling pathways (Aitken et al., 1998). A key step in the signaling pathways controlling sperm capacitation and the acrosome reaction is believed to be protein tyrosine phosphorylation (Baumber et al., 2003). Thus, third aim of this thesis was to study the interplay between ROS and protein phosphorylation in fish spermatozoa. Results of the current work confirmed that in spermatozoa of common carp tyrosine phosphorylation participates in stress response induced by X–XO (Chapter 4). Even low doses of free radicals were observed to alter the phosphorylation state of O-linked N-acetylglucosamine transferase (OGT) and septin-8-A, suggesting that these proteins are especially sensitive to ROS. OGT and septin-8-A were modified by all tested concentrations of X–XO, both in the presence and absence of antioxidant enzymes. The OGT activity is also involved in the acrosome reaction in mammalian spermatozoa as well as in cell-cell interaction during gamete fusion (Wu and Sampson, 2014). Thus the effect of ROS on the OGT phosphorylation suggests that in fish, as well as in mammalian spermatozoa, ROS are involved in processes of sperm-egg fusion, even though carp spermatozoa do not possess an acrosome. Hence further studies are needed to study cell structures involved in gamete interaction in carp and the role of ROS in this process.

Septin-8-A has been shown to be dephosphorylated under oxidative stress in carp spermatozoa (Chapter 4). A similar result was observed after carp and sterlet sperm motility activation in hypotonic conditions (Chapter 5). The role of septins in fish sperm motility is not clear yet. However, it can be hypothesized that microtubule-associated septins may be regulated by ROS and participate in sperm motility by spatially modulating the association of tubulin with post-translationally modifying enzymes and microtubule motors.

The role of protein phosphorylation in fish spermatozoa motility has been elucidated in the Chapter 5 of the present work. The number of axonemal proteins and signaling enzymes involved in spermatozoa motility of sterlet and carp were localised and identified in this study. Moreover, it has been shown that protein kinase A (PKA) and protein kinase C (PKC) participate in sperm motility of both species. Though the role of ROS in intracellular signaling during motility activation and regulation is not clear yet; previous studies have shown that PKC could participate in redox regulation (Lambert et al., 2003). It has been demonstrated that PKC promotes phosphorylation of p47phox, a component of the NADPH oxidase complex, and subsequently activation of the complex, which produce ROS in mammalian cells (Fontayne et al., 2002).

Several heat shock proteins were found in spermatozoa of carp (Chapter 5). HSP70 is one of the most important defence mechanisms of different cell types and plays an important role in protecting cells against a broad spectrum of potentially lethal pollutants (Gupta et al., 2010). The abundancy of heat shock proteins in carp spermatozoa allow us to suggest that in normal conditions spermatozoa after spawning are prepared to withstand a great osmotic and oxidative shock. Additionally carp spermatozoa possess a natural protection system against pollutants, which was not found in spermatozoa of sterlet. This result leads us to conclusion that spermatozoa of freshwater fish apply different pathways of stress response, which may depend on their adaptation to different environmental conditions.

Overall results of this thesis confirm that fish sperm provide an important test tool for toxicity studies and show great promise as a useful indicator of low level toxic effects. Our findings suggest that spermatozoa motility of freshwater fish is a complex process, regulated by post-translational protein modifications and various signaling molecules. Modulation of these pathways by environmental pollutants or ROS can lead to violations of motility parameters and drastically affect sperm fertilising ability.

CONCLUSIONS

In this thesis, which includes three publications, the effect of xenobiotics and ROS on sperm performance and intracellular signaling of chondrostean and teleostean fish is described. Additionally we discussed the role of protein phosphorylation, kinase activity and secondary messengers involved in sperm motility activation and regulation in these species. The main conclusions obtained from this work are:

1. Sturgeon spermatozoa are highly susceptible to the presence of endocrine disrupting chemicals in environment, which induce excessive ROS production even at low concentrations. Therefore the use of fish spermatozoa to detect toxic effect of water pollutants can be proposed as a novel mean of assessment of water quality.
2. ROS production has been shown to be deleterious to fish spermatozoa, reducing sperm motility and ATP content, inducing DNA damage, lipid and protein oxidation. However, catalase, superoxide dismutase, and glutathione in combination with seminal plasma can reduce oxidative stress in carp spermatozoa and improve sperm quality.
3. ROS alter intracellular signaling pathways by altering protein phosphorylation and phosphatase activity in carp spermatozoa.
4. Protein phosphorylation is involved in fish sperm motility activation, processes of energy metabolism and intracellular signaling. PKA and PKC play important role in motility activation of carp and sterlet spermatozoa.

REFERENCES

Aitken, R.J., Baker, M.A., 2006. Oxidative stress, sperm survival and fertility control. Mol. Cell. Endocrinol. 250, 66–69.

Aitken, R.J., Buckingham, D., Harkiss, D., 1993. Use of a xanthine-oxidase free-radical generating-system to investigate the cytotoxic effects of reactive oxygen species on human spermatozoa. J. Reprod. Fertil. 97, 441–450.

Aitken, R.J., Harkiss, D., Knox, W., Paterson, M., Irvine, D.S., 1998. A novel signal transduction cascade in capacitating human spermatozoa characterised by a redox-regulated, cAMP-mediated induction of tyrosine phosphorylation. J. Cell Sci. 111, 645–656.

Barranger, A., Akcha, F., Rouxel, J., Brizard, R., Maurouard, E., Pallud, M., Menard, D., Tapie, N., Budzinski, H., Burgeot, T., Benabdelmoun, A., 2014. Study of genetic damage in the Japanese oyster induced by anenvironmentally-relevant exposure to diuron: Evidence of verticaltransmission of DNA damage. Aquat. Toxicol. 146, 93–104.

Baumber, J., Sabeur, K., Vo, A., Ball, B.A., 2003. Reactive oxygen species promote tyrosine phosphorylation and capacitation in equine spermatozoa. Theriogenology 60, 1239–1247.

Fontayne, A., Dang, P.M., Gougerot-Pocidalo, M.A., El Benna, J., 2002. Phosphorylation of p47phox sites by PKC alpha, beta II, delta, and zeta: effect on binding to p22[phox] and on NADPH oxidase activation. Biochemistry 41, 7743–7750.

Griveau, J. F., Dumont, E., Renard, P., Callegari, J. P., Le Lannou D., 1995. Reactive oxygen species, lipid peroxidation and enzymatic defence systems in human spermatozoa. J. Reprod. Fertil. 103, 17–26.

Gupta, S.C., Sharma, A., Mishra, M., Mishra, R.K., Chowdhuri, D.K., 2010. Heat shock proteins in toxicology: how close and how far? Life Sci. 86, 377–384.

Hagedorn, M., McCarthy, M., Carter, V.L., Meyers, S.A., 2012. Oxidative stress in zebrafish (*Danio rerio*) sperm. PLoS ONE 7, e39397.

Hulak, M., Gazo, I., Shaliutina, A., Linhartová, P., 2013. *In vitro* effects of bisphenol A on the quality parameters, oxidative stress, DNA integrity and adenosine triphosphate content in sterlet (*Acipenser ruthenus*) spermatozoa. Comp. Biochem. Physiol. C Toxicol. Pharmacol. 158, 64-71.

Kodoma, H., Yamaguchi, R., Fukuda, J., Kasai, H., Tanaka, T., 1997. Increased oxidative deoxyribonucleic acid damage in the spermatozoa of infertile male patients. Fertil. Steril. 68, 519–524.

Koziorowska-Gilun, M., Koziorowski, M., Fraser, L., Strzeżek, J. 2011. Antioxidant defence system of boar cauda epididymidal spermatozoa and reproductive tract fluids. Reprod. Domest. Anim. 46, 527–533.

Lambert, I.H., 2003. Reactive oxygen species regulate swelling-induced taurine efflux in NIH3T3 mouse fibroblasts. J. Membrane Biol. 192, 19–32.

Li, P., Li, Z.H., Dzyuba, B., Hulak, M., Rodina, M., Linhart, O., 2010. Evaluating the impacts of osmotic and oxidative stress on common carp (*Cyprinus carpio*, L.) sperm caused by cryopreservation techniques. Biol. Reprod. 83, 852–858.

Linhartova, P., Gazo, I., Shaliutina, A., Hulak, M., 2013. The *in vitro* effect of duroquinone on functional competence, genomic integrity, and oxidative stress indices of sterlet (*Acipenser ruthenus*) spermatozoa. Toxicol. in Vitro 27, 1612–1619.

Mekhfi, H., Veksler, V., Mateo, P., Maupoil, V., Rochette, L., Ventura-Clapier, R., 1996. Creatine kinase is the main target of reactive oxygen species in cardiac myofibrils. Circ. Res. 78, 1016–1027.

Morales, M., Martínez-Paz, P., Ozáez, I., Martínez-Guitarte, J.L., Morcillo, G., 2013. DNA damage and transcriptional changes induced by tributyltin (TBT) after short in vivo exposures of *Chironomus riparius* (Diptera) larvae. Comp. Biochem. Physiol. C 158, 57–63.

Perchec, G., Jeulin, C., Cosson, J., André, F., Billard, R., 1995. Relationship between sperm ATP content and motility of carp spermatozoa. J. Cell Sci. 108, 747–753.

Rurangwa, E., Kime, D.E., Ollevier, F., Nash, J.P., 2004. The measurement of sperm motility and factors affecting sperm quality in cultured fish. Aquaculture 234, 1–28.

Shaliutina-Kolesova, A.; Gazo, I.; Cosson, J.; Linhart, O. 2014. Protection of common carp (*Cyprinus carpio* L.) spermatozoa motility under oxidative stress by antioxidants and seminal plasma. Fish Physiology and Biochemistry 40, 1771-1781.

Shiva, M., Gautam, A.K., Verma, Y., Shivgotra, V., Doshi, H., Kumar, S., 2011. Association between sperm quality, oxidative stress, and seminal antioxidant activity. Clin. Biochem. 44, 319–324.

Thomas, P., Doughty, K., 2004. Disruption of rapid, nongenomic steroid actions by environmental chemicals: Interference with progestin stimulation of sperm motility in Atlantic croaker. Environ. Sci. Technol. 38, 6328–6332.

Wu, L., Sampson, N.S., 2014. Fucose, mannose, and β-N-Acetylglucosamine glycopolymers initiate the mouse sperm acrosome reaction through convergent signaling pathways. ACS Chem. Biol. 9, 468–475.

Zhang, X.Z., Xie, P., Wang, W.M., Li, D.P., Shi, Z.C., 2008. Plasma biochemical responses of the omnivorous crucian carp (*Carassius auratus*) to crude cyanobacterial extracts. Fish Physiol. Biochem. 34, 323–329.

Zhou, B.S., Liu, W.H., Siu, W.H.L., O'Toole, D., Lam, P.K.S., Wu, R.S.S., 2006. Exposure of spermatozoa to duroquinone may impair reproduction of the common carp (*Cyprinus carpio*) through oxidative stress. Aquat. Toxicol. 77, 136–142.

ENGLISH SUMMARY

The role of reactive oxygen species and protein phosphorylation in fish spermatozoa

Ievgeniia Gazo

Spermatozoa of fishes that have external fertilization are particularly vulnerable to damage after releasing into aqueous environment where there are great alterations in composition of media. Factors that affect spermatozoan movement in the external medium are water pollutants, temperature, pH and osmotic conditions. Numerous studies have shown that these factors can induce the production of reactive oxygen species (ROS) and, consequently, produce oxidative stress in different cell types, including sperm. The duration of sperm motility in freshwater fish species is very short, varying from 30 sec to 3 min. Moreover, antioxidant protection is limited in spermatozoa because of the very small volume of cytoplasm. Therefore any ROS-induced damage can significantly impair fertilization. Even though numerous freshwater fish are among highly profitable aquaculture species and their reproduction mechanisms are objectives of different research investigations, our knowledge about the role of oxidative stress and ROS in their spermatozoa is rather limited, as well as data on spermatozoan intracellular signaling and its interaction with ROS.

The goal of the present study was to investigate the effects of oxidative stress on sperm performance and intracellular signaling, particularly the effect of pollutants occurring in water environment. In addition, the molecular mechanisms of stress response and motility activation for spermatozoa of two freshwater fish species (*Acipenser ruthenus* and *Cyprinus carpio* L.) were analyzed.

Our results showed that endocrine disrupting chemicals, such as vinclozolin, at environmentally relevant concentrations induced a dose-dependent reduction in the motility of sterlet (*Acipenser ruthenus*) spermatozoa and the velocity (Chapter 2). Increased levels of lipid oxidation (LO) and protein carbonylation (CP), as well as changes in antioxidant activity of superoxide dismutase (SOD) indicate the development of an oxidative stress in spermatozoa exposed to xenobiotic. Moreover, increased DNA fragmentation, as well as a reduction of the level of ATP, was observed in spermatozoa incubated *in vitro* with xenobiotics. These results demonstrated that sterlet spermatozoa are highly susceptible to pollutants, which induce excessive ROS production even at low concentrations. Although the antioxidant system apparently responds to the oxidative stress, it seems that the antioxidant capacity of fish sperm is not sufficient to prevent cell damage. Therefore, the use of sperm for *in vitro* assays may provide a novel and efficient mean for evaluating the effect of xenobiotics in an aquatic environment. However, studies on the interplay between ROS and intracellular signaling by proteomic methods in sturgeon spermatozoa could be complicated by the absence of sturgeon protein database and poor genome sequencing.

To evaluate the role of ROS production in fish sperm and protective properties of seminal plasma, additional studies were performed on the common carp (*Cyprinus carpio* L.) spermatozoa (Chapter 3–4). The ROS were generated in carp spermatozoa by *in vitro* incubation with xanthine – xanthine oxidase system (X-XO). A time- and dose-dependent reduction in spermatozoa motility and velocity was observed as well as increased LO, CP and DNA fragmentation. Moreover, it was shown that O-linked N-acetylglucosamine transferase and septin-8-A changed their phosphorylation state, and acid phosphatase activity decreased in response to oxidative stress (Chapter 4). Thus, these results demonstrated that ROS impaired the functional parameters of carp spermatozoa, and altered intracellular signaling pathways through changes in tyrosine phosphorylation and acid phosphatase activity. On the other

hand, catalase (CAT), superoxide dismutase (SOD), and glutathione (GTH) in combination with seminal plasma can reduce oxidative stress in carp spermatozoa and improve sperm quality.

Our next study was conducted to provide basic knowledge about the molecular mechanisms of motility activation in spermatozoa of two taxonomically distant fish species: sterlet (*Acipenser ruthenus*) and common carp (*Cyprinus carpio* L.) (Chapter 5). Proteomic methods were used in order to better understand how sperm cells are constructed and how the protein phosphorylation is involved in the very rapid activation of flagellar motility. It was shown that the pattern of protein phosphorylation and their localization differs significantly between these two species. Phosphorylations on serine and tyrosine residues, as well as protein kinase A (PKA) substrates play an important role in spermatozoan motility activation and regulation in both species. Moreover, results of mass spectrometry analysis indicated that the majority of PKA substrata involved in spermatozoa motility activation belong to mitochondrial complex and glycolysis pathway. Results of analysis using kinase inhibitor suggest that the protein kinase C (PKC) activity is necessary for sperm motility activation in carp and sterlet. Furthermore, dynein intermediate chain was identified as a PKC substrate in sterlet spermatozoa. However, despite these findings, the role of PKC in fish spermatozoa motility remains unclear. Numerous signaling proteins involved in carp and sterlet spermatozoa movement were identified in this study, giving a better understanding of molecular mechanisms underlying sperm motility.

In conclusion, the results of this study provide new data on the effect of xenobiotics and oxidative stress on fish spermatozoa motility, DNA integrity, lipid and protein oxidation, antioxidant defense system and intracellular signaling. These data demonstrated the toxicity of water pollutants and ROS for fish spermatozoa and proposed the use of CAT, SOD, or GTH in combination with seminal plasma to reduce oxidative stress in these cells. Moreover, we identified many spermatozoan proteins that are involved in stress response and motility. In practice, the data presented in this thesis could be useful for elaboration of suitable medium for cryopreservation and artificial propagation of freshwater fish species.

CZECH SUMMARY

Vliv reaktivních forem kyslíku a proteinové fosforylace na funkci spermií ryb

Ievgeniia Gazo

Sperma ryb s externím oplozením je zvláště citlivé na poškození po vstupu do vodního prostředí, kde jsou velké změny ve složení média. Faktory, které ovlivňují pohyb spermií ve vnějším prostředí, jsou zejména vodní polutanty, teplota vody, pH a osmotický tlak. Mnoho studií prokázalo, že tyto faktory mohou vyvolat produkci reaktivních forem kyslíku (ROS) a v důsledku toho vytvářet oxidační stres v různých typech buněk, a to včetně spermií. Délka motility spermií sladkovodních ryb je velmi krátká a pohybuje se od 30 sekund do 3 minut. Kromě toho je antioxidační ochrana spermií omezena kvůli malému objemu jejich cytoplazmy, a proto by jakékoliv poškození indukované ROS mohlo významně narušit plodnost ryb. I přesto, že mnoho sladkovodních druhů ryb patří mezi vysoce rentabilní akvakulturní druhy a jejich reprodukční mechanizmy jsou cílem různých výzkumů, jsou naše poznatky o roli oxidačního stresu a ROS u spermií dosti omezené stejně jako údaje o spermatozoální intracelulární signalizaci a její interakci s ROS.

Cílem této studie bylo zjistit vliv oxidativního stresu na funkci spermií a na intracelulární signalizaci indukované různými znečišťujícími látkami vyskytujícími se ve vodním prostředí. Kromě toho byly analyzovány molekulární mechanizmy na stresovou reakci a aktivační proces pohyblivosti spermií u dvou druhů sladkovodních ryb – jeseter malý (*Acipenser ruthenus*) a kapr obecný (*Cyprinus carpio* L.).

Výsledky ukazují, že endokrinně disruptivní chemické látky jako Vinclozolin mohou vyvolat snížení pohyblivosti i rychlosti spermií jesetera malého, a to již při koncentracích vyskytujících se přirozeně ve vodním prostředí (kapitola 2) Zvýšená hladina lipidové oxidace (LO) a proteinové karbonylace (CP), stejně jako změna v antioxidačním účinku superoxiddismutázy (SOD), naznačují vznik oxidačního stresu u spermií, které byly předem vystaveny xenobiotikům. Kromě toho byla pozorována zvýšená fragmentace DNA a snížená úroveň ATP u spermií, které byly ovlivněny xenobiotiky *in vitro*. Výsledky ukazují, že sperma jesetera malého je velmi citlivé na přítomnost polutantů a již při nízkých koncentracích indukují nadměrnou produkci ROS. Ačkoliv antioxidační systém zřejmě reaguje na antioxidační stres, zdá se, že antioxidační kapacita rybího spermatu není dostatečná na to, aby zabránila poškození buněk. Z tohoto důvodu by použití spermatu v *in vitro* testech mohlo poskytnout nový a efektivní prostředek pro vyhodnocování účinku xenobiotik ve vodním prostředí. Bohužel studie o vzájemném vztahu mezi ROS a intracelulární signalizací proteomickou metodou u spermií jesetera jsou komplikované z důvodu absence databáze jeseteřích bílkovin a náročnosti sekvenace jeho genomu.

K vyhodnocení úlohy produkce ROS u rybích spermií a protektivních vlastností semenné plazmy byla další studie prováděna na spermiích kapra obecného (kapitoly 3–4). ROS byly generovány u kapřích spermií *in vitro* inkubací s xanthin – xanthinoxidázovým systémem (X–XO). Dávka škodlivé látky a doba působení měla vliv na snížení pohyblivosti a rychlosti spermií, stejně tak byla pozorována zvýšená hladina LO, CP a DNA fragmentace. Navíc bylo prokázáno, že reaktivní forma kyslíku vázaná na N-acetylglukosaminovou transferázou a septin-8-A změnila stav jejich fosforylace a aktivita acid-fosfatázy se snížila v reakci na oxidativní stres. Bylo rovněž zjištěno, že ROS zhoršují funkční parametry kapřích spermií a způsobují změny v intracelulárních signálních dráhách prostřednictvím změn fosforylace tyrosinu a kyseliny fosforečné. Na druhou stranu, kataláza (CAT), superoxiddismutáza (SOD) a glutathion (GTH) v kombinaci se semennou plazmou můžou mít vliv na snížení oxidačního stresu spermatu kapra a zlepšení kvality spermií.

   Další studie byla provedena s cílem získat základní znalosti o molekulárních mechanizmech aktivace pohyblivosti spermií u dvou taxonomicky vzdálených druhů ryb: jesetera malého a kapra obecného (kapitola 5). Proteomické metody posloužily k porozumění, jak jsou buňky spermií konstruovány a jak se fosforylační bílkoviny podílejí na velmi rychlé aktivaci pohyblivosti bičíku. Ukázalo se, že typ proteinové fosforylace a její lokalizace se značně liší mezi oběma druhy ryb. Fosforylace na serinovém a tyrosinovém zbytku, stejně jako protein-kináza A (PKA), hrají důležitou roli u pohyblivosti, aktivace a regulace spermií obou druhů. Navíc výsledky analýz hmotnostní spektrometrií ukázaly, že většina substrátů PKA podílejících se na aktivaci pohyblivosti patří do komplexu mitochondriální a glykolýzové dráhy. Kromě toho dyneinový střední řetězec byl identifikován jako PKC substrát spermií jesetera malého. Úloha PKC u rybích spermií zůstává zatím nejasná. Mnoho signalizačních proteinů, které byly identifikovány v této studii a které se podílejí na pohybu spermií kapra obecného a jesetera malého, nám lépe objasňují molekulární mechanizmy pohybu spermií.

   Závěrem lze říci, že výsledky této práce nám poskytují nové údaje o vlivu xenobiotik a oxidačního stresu na pohyblivost rybích spermií, integritu DNA, lipidovou a proteinovou oxidaci, antioxidační obranný systém a intracelulární signalizaci. Naše výsledky prokázaly toxicitu některých vodních polutantů a vliv ROS na rybí spermie stejně jako blahodárný účinek CAT, SOD nebo GTH v kombinaci se semennou plazmou na snížení oxidačního stresu spermií. Navíc jsme identifikovali mnoho spermiálních proteinů podílejících se na stresové reakci a pohyblivosti. V praxi by mohla být uvedená data v této práci užitečná pro vypracování vhodného média pro kryokonzervaci a umělý výtěr sladkovodních druhů ryb.

ACKNOWLEDGMENTS

5. GAJU 046/2010/Z – Reproduction and genetics of selected model species of teleostean and chondrostean fish (2010–2012, responsible leader PProf. Otomar Linhart)

6. GACR P502//12/1973 – Characterization of swimming fish sperm flagella: biophysical quantification (2012–2015, responsible leader Jacky Cosson, Ph.D., Dr. h. c.)

7. GACR P502/11/0090 – Maturation and ageing of fish spermatozoa: A comparative study between teleostean and chondrostean fish species as taxonomically distant models (2011–2015, responsible leader Marek Rodina, Ph.D.)

8. GACR P502/15/12034S –Ancient mechanism of sturgeon sperm for acquisition of ability to fertilize (2015–2017, responsible leader Jacky Cosson, Ph.D., Dr. h. c.)

9. GACR 14-02940S – Ploidy and hybrid diversity in sturgeons (Acipenseriformes) and its impacts on conservation and breeding (2014–2016, responsible leader Prof. Martin Flajšhans)

10. GACR 523-08-0824 – Relationships of ploidy level, genome and cell size in model polyploid fish with cytological and physiological impacts on conservation and culture (2008–2012, responsible leader Prof. Martin Flajšhans)

11. IAA 608030801 – Diversity of bioenergetics pathways, membrane functions, signalling mechanisms and proteomics of cryopreserved sperm of evolutionary different fish species (2008–2012, responsible leader Prof. Otomar Linhart)

12. ME10015 – The using of sex reverse and proteomic analisis in frozen sperm of paddlefish for production of caviar (2010–2012, responsible leader Prof. Otomar Linhart)

13. QH82119 – Research of sperm and embryos cryopreservation (2008–2012, responsible leader Prof. Otomar Linhart)

14. COST (No LD14119) – Aquatic gametes as model for fundamental cell motility studies (2014–2016, responsible leader Martin Pšenička, Ph.D.)

15. STSM-FA1205-16781

16. STSM-FA1205-20685

LIST OF PUBLICATIONS

Peer – reviewed journals with IF

Gazo, I., Shaliutina-Kolesova, A., Dietrich, M.A., Linhartová, P., Shaliutina, O., Cosson, J. 2015. The effect of reactive oxygen species on motility parameters, DNA integrity, tyrosine phosphorylation and phosphatase activity of common carp (*Cyprinus carpio* L.) spermatozoa. Molecular Reproduction and Development 82: 48–57.

Linhartová, P., **Gazo, I.,** Shaliutina-Kolešová, Hulák, M., Kašpar, V., 2015. Effects of tetrabrombisphenol A on dna integrity, oxidative stress, and sterlet (*Acipenser ruthenus*) spermatozoa quality variables. Environmental Toxicology 30: 735–745.

Pšenička, M., Saito, T., Linhartová, Z., **Gazo, I.,** 2015. Isolation and transplantation of sturgeon early-stage germ cells. Theriogenology 83: 1085–1092.

Dzyuba, B., Cosson, J., Boryshpolets, S., Bondarenko, O., Dzyuba, V., Prokopchuk, G., **Gazo, I.,** Rodina, M., LInhart, O., 2014. *In vitro* sperm maturation in sterlet, *Acipenser ruthenus*. Reproductive Biology 14: 160–163.

Lebeda, I., **Gazo, I.,** Flajshans, M., 2014. Chemical induction of haploid gynogenesis in sterlet *Acipenser ruthenus*. Czech Journal of Animal Science 59: 310–318.

Linhartová, Z., Rodina, M., Guralp, H., **Gazo, I.,** Saito, T., Pšenička, M., 2014. Isolation and cryopreservation of early stages of germ cells of tench (*Tinca tinca*). Czech Journal of Animal Science 59: 381–390.

Shaliutina-Kolesova, A., **Gazo, I.,** Cosson, J., Linhart, O., 2014. Protection of common carp (*Cyprinus carpio* L.) spermatozoa motility under oxidative stress by antioxidants and seminal plasma. Fish Physiology and Biochemistry 40: 1771–-1781.

Gazo, I., Linhartová, P., Shaliutina, A., Hulák, M., 2013. Influence of environmentally rele-vant concentrations of vinclozolin on quality, DNA integrity, and antioxidant responses of sterlet *Acipenser ruthenus* spermatozoa. Chemico-Biological Interactions 203: 377–385.

Hulák, M., **Gazo, I.,** Shaliutina, A., Linhartová, P., 2013. *In vitro* effects of bisphenol A on the quality parameters, oxidative stress, DNA integrity and adenosine triphosphate content in sterlet (*Acipenser ruthenus*) spermatozoa. Comparative Biochemistry and Physiology – Part C: Toxicology & Pharmacology 158: 64–71.

Linhartová, P., **Gazo, I.,** Shaliutina, A., Hulak, M., 2013. The *in vitro* effect of duroquinone on functional competence, genomic integrity, and oxidative stress indices of sterlet (*Acipenser ruthenus*) spermatozoa. Toxicology in Vitro 27: 1612–1619.

Shaliutina, A., Hulák, M., **Gazo, I.,** Linhartová, P., Linhart, O., 2013. Effect of short-term storage on quality parameters, DNA integrity, and oxidative stress in Russian (*Acipenser gueldenstaedtii*) and Siberian (*Acipenser baerii*) sturgeon sperm. Animal Reproduction Science 139: 127–135.

Shaliutina-Kolešová, A., **Gazo, I.,** Cosson, J., Linhart, O., 2013. Comparison of oxidant and antioxidant status of seminal plasma and spermatozoa of several fish species. Czech Journal of Animal Science 58: 313–320.

Abstracts and conference proceedings

Gazo, I., Linhartova, P., Shaliutina-Kolesova, A., Cosson, J., 2013. Bisphenol A effects on protein profile and DNA integrity of spermatozoa of brook trout (*Salvelinus fontinalis*). In: Book of abstracts. Diversification in Inland Finfish Aquaculture II (DIFA II), 24–26 September, 2013, Vodnany, Czech Republic.

Gazo, I., Shaliutina, A., Linhartova, P., Cosson, J., 2013. The effect of reactive oxygen species on protein phosphorylation pattern in spermatozoa of common carp (*Cyprinus carpio*). In: Book of abstracts. 4th Workshop on the Biology of Fish Gametes, 17–20 September, 2013, Algarve, Portugal.

Gazo, I., Linhartova, P., Shaliutina, A., Hulak, M., 2012. The effect of environmentally related concentrations of vinclozolin on DNA integrity and oxidative stress indices in sterlet (*Acipenser ruthenus*) sperm. In: Book of abstracts. AQUA2012, 1–5. September, 2012, Prague, Czech Republic.

TRAINING AND SUPERVISION PLAN DURING STUDY

Name	Ievgeniia Gazo
Research department	2011–2015 – Laboratory of Mollecular, Cellular and Quantitative Genetics of FFPW
Supervisor	Jacky Cosson Ph.D., Dr. h. c.
Period	3rd October 2011 until 14th September 2015

Ph.D. courses	Year
Basic of scientific communiacation	2012
Pond aquaculture	2012
Applied hydrobiology	2012
Ichthyology and systematic of fish	2013
English Language	2014

Scientific seminars	Year
Seminar days of RIFCH and FFPW	2012 2013 2014 2015

International conferences	Year
Gazo, I., Linhartova, P., Shaliutina, A., Hulak, M., 2012. The effect of environmentally related concentrations of vinclozolin on DNA integrity and oxidative stress indices in sterlet (*Acipenser ruthenus*) sperm. AQUA2012, Prague, Czech Republic, 1–5 September, 2012. (*oral presentation*)	2012
Gazo, I., Shaliutina, A., Linhartova, P., Cosson, J., 2013. The effect of reactive oxygen species on protein phosphorylation pattern in spermatozoa of common carp (*Cyprinus carpio*). 4th Workshop on the Biology of Fish Gametes, 17–20 September, 2013, Algarve, Portugal. (*oral presentation*)	2013
Gazo, I., Linhartova, P., Shaliutina-Kolesova, A., Cosson, J., 2013. Bisphenol A effects on protein profile and DNA integrity of spermatozoa of brook trout (*Salvelinus fontinalis*). Book of abstracts. Diversification in Inland Finfish Aquaculture II (DIFA II), 24–26 September, 2013, Vodnany, Czech Republic. (*poster presentation*)	2013

Foreign stays during Ph.D. study at RIFCH and FFPW	Year
Prof. Kazuo Inaba, Tsukuba University, Shimoda Marine Research Center, Shimoda, Japan. (1 month, studies on molecular mechanism of sperm flagellar motility in marine invertebrates)	2013
Prof. Andrzej Ciereszko, Institute of Animal Reproduction and Food Research, Polish Academy of Sciences, Department of Gamete and Embryo Biology, Olsztyn, Poland. (1 month, study of proteomic techniques)	2013
Dr. Janet Chenevert, Marine Station in Villefranche-sur-Mer, Université Pierre et Marie Curie, France. (1 month, molecular mechanisms of motility activation and intracellular signaling in spermatozoa of different fish species: sterlet (*Acipenser ruthenus*) and common carp (*Cyprinus carpio* L.)	2014
Dr. Remi Dumollard, Marine Station in Villefranche-sur-Mer, Université Pierre et Marie Curie, France. (6 weeks, the carcinogenic and reprotoxic effects of endocrine disruptors on eggs and embryo development of tunicate (*Phallusia mammillata*)	2014

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EDUCATION

Oct. 2011 – present: Ph.D. student in Fishery, Faculty of Fisheries and Protection of Waters, University of South Bohemia in České Budějovice, Vodňany, Czech Republic

2010–2011 M.Sc., Faculty of Radiophysics, Department of Biological and Medical Physics, V. N. Karazin Kharkiv National University, Kharkiv, Ukraine

2006–2010 B.Sc., Faculty of Radiophysics, Department of Biological and Medical Physics, V. N. Karazin Kharkiv National University, Kharkiv, Ukraine
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2014 – Grant Agency of the University of South Bohemia: GAJU 016/2014/Z. Molecular mechanisms of motility activation and intracellular signaling in spermatozoa of different fish species: sterlet (*Acipenser ruthenus*) and common carp (*Cyprinus carpio* L.)

COMPLETED COURSES

2014 – Course on Biological Specimen Preparation for Electron Microscopy – Biology Centre, Academy of Sciences, Laboratory of Electron Microscopy, České Budějovice, Czech Republic

2013 – Microscopy and Image Analysis workshop – Faculty of Fisheries and Protection of Waters, University of South Bohemia, Vodňany, Czech Republic

2012 – Basics of scientific communication – Faculty of Fisheries and Protection of Waters, University of South Bohemia, Vodňany, Czech Republic

RESEARCH STAY AND COLLABORATIONS

22. 09. – 31. 10. 2014 – Dr. Remi Dumollard, Marine Station in Villefranche-sur-Mer, Université Pierre et Marie Curie, France

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