Protein phosphorylation in spermatozoa motility of *Acipenser ruthenus* and *Cyprinus carpio*

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**Summary**

Spermatozoa of externally fertilizing freshwater fish possess several different modes of motility activation. Spermatozoa of common carp (*Cyprinus carpio* L.) are activated by hypoosmolality, whereas spermatozoa of sterlet (*Acipenser ruthenus*) require Ca²⁺ and low concentration of K⁺ for motility activation. Intracellular signaling differs between these two species as well, particularly in terms of utilization of secondary messengers (cAMP and Ca²⁺), and kinase activities. The current study was performed in order to determine the importance of protein phosphorylation and protein kinases for activation of sperm motility in carp and sterlet. Treatment with kinase inhibitors indicates that protein kinases A and C (PKA and PKC) participate in spermatozoa motility of both species. Immunodetection of phospho-(Ser/Thr) PKA substrates shows that phosphorylated proteins are localized differently in spermatozoa of carp and sterlet. Strong phosphorylation of PKA substrate was observed in flagella of sterlet spermatozoa, whereas in carp sperm, PKA substrates were lightly phosphorylated in the midpiece and flagella. Motility activation induced either phosphorylation or dephosphorylation on serine, threonine and tyrosine residues of numerous proteins in carp and sterlet spermatozoa. Proteomic methods were used to identify proteins whose phosphorylation state changes upon the initiation of sperm motility. Numerous mitochondrial and glycolytic enzymes were identified in spermatozoa of both species, as well as axonemal proteins, heat shock proteins, septins and calcium-binding proteins. Our results contribute to an understanding of the roles of signaling molecules, protein kinases and protein phosphorylation in motility activation and regulation of two valuable fish species, *C. carpio* and *A. ruthenus*.

**Introduction**

Spermatozoa motility represents a very important prerequisite leading to the contact and fusion between male and female gametes for fertilization. The development of spermatozoa motility is a complex process with two phases: a very rapid response to activating triggers, which occurs in the range of milliseconds, followed by a maintenance phase which involves a network of signaling pathways providing energy that lasts from a few seconds to several hours depending on the species (Dzyuba et al. 2015). In some freshwater fish species, such as the carp (*Cyprinus carpio*), very low water osmolality causes spermatozoa motility activation (Perchec Poupad et al. 1997), whereas in sturgeons activation can be achieved without osmolality variation (as compared with seminal fluid) through changes in ionic composition of the surrounding medium (Alavi & Cosson 2006). Extracellular factors controlling sperm motility act on the flagellar motile apparatus, the axoneme, through signal transduction, cAMP and Ca²⁺ serving as second messengers. Mechanisms of intracellular signaling also vary among different species: in carp spermatozoa, motility activation does not require increased cAMP concentration (Krasznai et al. 2000); however, spermatozoa of sterlet depend on cAMP and Ca²⁺ ions for motility activation (Linhart et al. 2002, Alavi et al. 2011).

Since protein synthesis is switched off in spermatozoa before maturation (Baker 2016), one of the most important processes in sperm intracellular signaling involves post-translational modifications such as protein phosphorylation, which was extensively studied in human spermatozoa (O’Flaherty et al. 2006). Phosphorylation of proteins on serine/threonine (Ser/Thr) and tyrosine (Tyr) residues is controlled by the activity of protein kinases, while dephosphorylation of these residues is mediated by protein phosphatases. The balance of the kinase...

The second messengers, cAMP and calcium, affect sperm motility by regulating the cAMP-dependent protein kinase A (PKA) and the Ca2+-dependent protein kinase C (PKC) respectively. The contribution of PKA as a regulator of spermatozoa motility initiation was described for numerous species (Brokaw 1987, Inaba 2003). In salmonids, 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). In spermatozoa, one of the major known targets of this kinase is dynein (Hamasaki et al. 1991), a microtubule-dependent force-generating ATPase, which plays a key role in axonemal microtubule sliding (Visconti et al. 1995, Inaba 2003). The regulation of dynein-mediated sliding of the axonemal microtubule outer-doublet through protein phosphorylation/dephosphorylation by PKA has been described for different mammalian species (Lindemann & Kanous 1989), as well as for rainbow trout, chum salmon, sea urchin (Inaba et al. 1999) and tunicate (Nomura et al. 2000). PKA is also involved in signaling processes on the outer side of 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 for several fish species (Hayashi et al. 1987, Morita et al. 2003). However, it is not clear whether PKA is not involved in motility activation of those species or whether its activity is regulated by another pathway (Kohr et al. 2010). PKC, a serine/threonine kinase, has a key role during initiation and maintenance of motility in sea urchin spermatozoa (White et al. 2007). Conventional PKC isoforms have a requirement for Ca2+ for their activation (Newton 1995). Thus, activity of PKC is strongly associated with and controlled by Ca2+ signaling.

In addition to motility activation, fertilization processes regulated by protein phosphorylation include capacitation and the acrosome reaction in mammals, all of which are required for spermatozoan/egg fusion (O’Flaherty et al. 2006). Aside from dyneins, a few other phospho-proteins participating in fish sperm motility have been described (Inaba et al. 1998, Itoh et al. 2003, Zilli et al. 2016) but most of them are yet to be characterized. In particular, there is a lack of knowledge about the role of protein phosphorylation in spermatozoa of commercially important aquaculture fish species such as the sterlet (Acipenser ruthenus, a small sturgeon) and the common carp (Cyprinus carpio L.). In the present study, we address the changes in protein phosphorylation pattern associated with spermatozoa motility and identify potential kinase substrates in spermatozoa of these two species.

Materials and methods

Reagents

Protein kinase inhibitors staurosporine, H-89, chelerythrine, Gö6983 and tyrophostin 23 were purchased from the Sigma-Aldrich Co. ReadyStrip IPG strips, protein markers and polyvinylidene difluoride (PVDF) membranes were from Bio-Rad laboratories. Anti-phosphotyrosine, anti-phosphoserine, anti-phosphothreonine and anti-PKC polyclonal antibodies produced in rabbit were from Abcam. Phospho-(Ser/Thr) PKA substrate, phospho-(Ser) PKC substrate and PKA C-α polyclonal antibodies produced in rabbit and HRP-conjugated goat anti-rabbit IgG were purchased from Cell Signaling Technology (Biotech A.S., Prague, Czech Republic).

Ethics

Manipulations with animals were performed according to authorization for breeding and delivery of experimental animals (Reference number: 44218/2015-MZE-17214 17OZ14202/2015-17214, valid from 17th August 2015 for 5 years) and the authorization for the use of experimental animals (Reference number: 2293/2015-MZE-17214 16OZ22302/2014-17214, valid from 22th January 2015 for 5 years) issued to the Faculty of Fisheries and Protection of Waters, University of South Bohemia by Ministry of Agriculture of the Czech Republic.

Broodstock handling and sperm collection

Sperm was obtained from adult common carp (Cyprinus carpio L., n = 6; body weight: 2–3 kg) and sterlet (Acipenser ruthenus, 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. Before 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 body weight for carp and 5 mg/kg of body weight for sterlet, 24 h 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, feces 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 20x using a Burker cell hemocytometer. Average spermatozoa concentration was 55.1 ± 2.2 × 10⁹/mL for carp and 1.26 ± 0.7 × 10⁹/mL for sterlet.

Inhibitor treatment

The following inhibitors were used: staurosporine (broad spectrum inhibitor of protein kinases), H-89 (PKA inhibitor), chelerythrine and Gö6983 (inhibitors of PKC) and tyrophostin 23 (PKT inhibitor). Separate sperm samples from each

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Protein phosphorylation in fish sperm motility

Experimental male were centrifuged at 300 g 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 and coworkers and contained 200 mM KCl, 30 mM Tris–HCl, pH 8.0; IM for sterlet contained 20 mM Tris, 30 mM NaCl and 2 mM KCl, pH 8.5. Sperm was diluted to a final concentration of 5 x 10^8 cells/mL (Perchec et al. 1995). Protein kinase inhibitors were first dissolved in dimethyl sulfoxide (DMSO) at 100 mM, except for chelerythrine, which was dissolved at 10 mM, and then diluted to desired concentrations with IM, so that the final concentration of DMSO in incubation medium did not exceed 1%. The sperm samples were pre-incubated at 4°C for 5 min with the inhibitors in IM, and then activated, as described below, in 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 CaCl2, pH 8.5 for sterlet; motility was recorded and analyzed as described below.

**Spermatozoa motility analysis**

The percentage of motile spermatozoa was determined after triggering sperm motility under dark-field microscopy (Olympus BX 50, Japan). For triggering motility, sperm in IM was diluted with AM at a final dilution of 1:5000 and then a drop was placed on a microscope slide. To prevent spermatozoa from sticking to the 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 strobescope flash (ExposureScopeTM). The movements of the spermatozoa heads were analyzed using Olympus MicroImage software (version 4.0.1. for Windows with a special macro by Olympus C & S). Briefly, spermatozoa head positions on five successive frames are assigned different colors: frame 1 red, frames 2–4 green and frame 5 blue. Those that moved were visible in three colors, while non-moving spermatozoa were white. The percent motile was calculated at 15 s post activation from the number of white and red cells. 20–40 spermatozoa were counted for each frame. Spermatozoa motility activation and measurement were obtained in triplicate for each sample.

**Sample preparation and protein extraction**

Samples for Western blotting and for two-dimensional protein analysis were prepared as follows: sperm pellets were diluted either with IM or with 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 CaCl2, pH 8.5 for sterlet; motility was recorded and analyzed as described below.

Spermatozoa were washed three times with TBST, and then incubated for 16 h at 4°C with polyclonal antibodies to either phosphotyrosine, phosphoserine, a phosphothreonine, PKA, PKC, phospho-(Ser/Thr) PKA substrates or phospho-(Ser) PKC substrates diluted 1:1000 in 5% (w/v) 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% w/v EDTA, 1 mM okadaic acid) and protease inhibitors (100 mM PMSF, 1 mg/mL pepstatin 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, 25 µg of protein was loaded for each lane. Sperm proteins were added to sample buffer containing 65 mM Tris, 10% (v/v) glycerol, 2% (w/v) SDS, 5% (v/v) beta-mercaptoethanol and 1% bromophenol blue, and then heated for 3 min at 95°C. Proteins were separated on a 12% acrylamide gel using the Bio-Rad Mini-PROTEAN vertical electrophoresis system.

**Two-dimensional electrophoresis**

For two-dimensional (2D) electrophoresis, proteins were extracted from immotile or from activated spermatozoa (30 s post activation for carp sperm and 120 s for sterlet). The protein concentration was determined as described previously. Isoelectric focusing was done 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 (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 h; isoelectric focusing, 500 V for 1 h, 1000 V for 1 h, 3000 V for 1 h (gradient) and 8000 V for 2 h (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% iodoacetamide instead of dithiothreitol for a further 15 min. The strip was loaded in 0.5% agarose in running buffer for SDS-PAGE poured over a 12% acrylamide gel, and the proteins separated according to molecular weight by electrophoresis. Some gels were further used for Western blot analysis, others were stained with Coomassie Brilliant Blue R-250 to visualize protein spots.

**Western blot analysis**

After SDS-PAGE and 2D electrophoresis, gels were placed on polyvinylidene difluoride (PVDF) membranes and electrically transferred. The total amount of protein loaded and transferred was controlled before immunodetection by staining the membrane with 0.1% (w/v) 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. The membranes were then blocked by incubation with 5% (w/v) non-fat milk in TBST (0.1% v/v Tween-20, 20 mM Tris, 65 mM Tris, 10% (v/v) glycerol, 2% (w/v) SDS, 5% (v/v) beta-mercaptoethanol and 1% bromophenol blue, and then heated for 3 min at 95°C. Proteins were separated on a 12% acrylamide gel using the Bio-Rad Mini-PROTEAN vertical electrophoresis system.
I Gazo and others, see section on ′ given at the end of this article).

Table 1 P′(2017) α Supplementary Fig. 1). Reproduction (2093.086), ACTH clip 18 (2465.19) and somatostatin 28 consisting of angiotensin II (1046.54), angiotensin I (1296.68), [M+H]+ ion peptide calibration standards (Bruker Daltonics) Germany). The MS and MS/MS LIFT spectra of selected ions were analyzed on a time-of-flight Autoflex-TOF/TOF mass spectrometer (MALDI-TOF-TOF, Bruker Daltonics, Bremen, Germany). Peptide samples were loaded on the Zip-Tip and contaminants had been first equilibrated by sequential washings with 100% acetonitrile, 50% acetonitrile-0.1% TFA and 0.1% TFA in water. The sample was loaded on the Zip-Tip and contaminants had been first equilibrated by sequential washings with 100% acetonitrile, 50% acetonitrile-0.1% TFA and 0.1% TFA in water. The sample was loaded on the Zip-Tip and contaminants had been first equilibrated by sequential washings with 100% acetonitrile, 50% acetonitrile-0.1% TFA and 0.1% TFA in water. The sample was loaded on the Zip-Tip and contaminants had been first equilibrated by sequential washings with 100% acetonitrile, 50% acetonitrile-0.1% TFA and 0.1% TFA in water. The sample was loaded on the Zip-Tip and contaminants had been first equilibrated by sequential washings with 100% acetonitrile, 50% acetonitrile-0.1% TFA and 0.1% TFA in water.

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% v/v acetonitrile in 50mM (NH4)HCO3 for 15 min. The gel pieces were treated with 5 mL of 50mM (NH4)HCO3 containing 1 μg of modified sequencing-grade trypsin and incubated for 12 h at 37°C. After digestion, the gel pieces were immersed in 100μL of 0.1% v/v trifluoroacetic acid (TFA) in water for further peptide extraction. The peptides were concentrated and desalted using Zip-Tip pipette tips (Millipore), which had been first equilibrated by sequential washings with 100% acetonitrile, 50% acetonitrile-0.1% TFA and 0.1% TFA in water. The sample was loaded on the Zip-Tip and contaminants were washed away 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 mixed with 1 μL of freshly made CHCA matrix and spotted on a steel MALDI target plate (MT 34 Target Plate Ground Steel, Bruker Daltonics). Peptide samples were analyzed on a time-of-flight Autoflex-TOF/TOF mass spectrometer (MALDI-TOF-TOF, Bruker Daltonics, Bremen, Germany). The MS and MS/MS LIFT spectra of selected ions were collected and calibrated externally using monoisotopic [M+H]+ ion peptide calibration standards (Bruker Daltonics) consisting of angiotensin II (1046.54), angiotensin I (1296.68), substance P (1347.73), bombesin (1619.82), ACTH clip 1 (2093.086), ACTH clip 18 (2465.19) and somatostatin 28 (3147.471). The MS peptide mass fingerprint (PMF) and fragment mass spectra (MS/MS) from each individual spot were combined and used to search against the NCBI database using MascotServer (Matrix Sciences) with the following settings: cleavage enzyme: trypsin, max missed cleavages: 2 and mass tolerance mono: 50 ppm, fragment ion mass tolerance of 0.5 Da; parent ion mass tolerance of 200 ppm; alkylation of cysteine by carbamidomethylation as a fixed modification and oxidation of methionine as a variable modification. For the PMF and MS/MS ion search, statistically significant (P ≤ 0.05) matches by MASCOT were regarded as correct hits.

Immunolabeling

Motility was activated by adding AM to sperm in Eppendorf tubes at a dilution ratio of 1:50. After activation, spermatozoa were attached to slides pre-coated with 0.01% w/v poly-l-lysine. After incubation for 1 min at 20°C, the sperm were fixed with 4% formaldehyde in IM for 30 min and stored in a moist chamber. The fixed 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 antibodies to phosphotyrosine, phosphoserine, phosphothreonine, PKA, PKC, phospho-(Ser/Thr) PKA substrates or phospho-(Ser) PKC substrates (dilution of 1:100) in the blocking buffer for 16 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 (Jackson ImmunoResearch Laboratories) at 1:200 dilution ratio for 2 h at 20°C, washed with TBST (5 min) at 20°C and mounted in 50% v/v glycerol or in certain cases treated with TBST containing 1 μM 4,6-diamidino-phenylindole (DAPI) before mounting. Slides were examined by fluorescent microscopy (Olympus BX50) at 60× magnification.

In-membrane digestion and mass spectrometry

The inhibitor of tyrosine kinase, tyrphostin 23 had no significant effect on spermatozoa motility in both species at all tested concentrations. On the other hand, treatment with the PKC inhibitor chelerythrine, inhibited motility of both species at 100μM, and significantly decreased motility of carp spermatozoa starting from a concentration of 50μM. In contrast, carp sperm motility was only barely affected at 100μM (17.5% inhibition compared to control) and decreased slightly at higher concentrations of inhibitor (Table 1).

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Protein phosphorylation in fish sperm motility

Changes in protein phosphorylation status and subcellular localization of phospho-proteins after motility activation in carp and sterlet spermatozoa

PKC substrates

Proteins were extracted from spermatozoa before and after motility activation and analyzed by immunoblot. Immunodetection with anti-PKC antibody confirmed the presence of PKC in spermatozoa of both species at 76 kDa that correspond to PKC β isoform (Fig. 1A). The antibody to phospho-(Ser) PKC substrates revealed that in sterlet spermatozoa, a protein of 110 kDa was phosphorylated in immotile spermatozoa and during the entire motility period, decreasing slowly over time (Fig. 2A). In the presence of PKC inhibitor chelerythrine, this band was dephosphorylated in immotile spermatozoa (Fig. 2A, left lane). In carp, one protein of 63 kDa was detected by the same antibody in immotile spermatozoa, disappeared after motility activation, and became phosphorylated again after 30 s of motility period (Fig. 2B). Chelerythrine completely blocked the phosphorylation of this band in immotile spermatozoa. Immunostaining of sterlet spermatozoa showed that the phospho-(Ser) PKC substrate antibody labeled flagella and midpiece (Fig. 2C). A similar distribution of phosphorylated protein was observed in carp spermatozoa using this antibody; however, the intensity of staining was lower (Fig. 2D). Immunostaining with anti-PKC antibody (Fig. 1C) showed that localization of the kinase matches the localization of phosphorylated substrates (Fig. 2C and D).

PKA substrates

Protein kinase A was detected in spermatozoa of sterlet and carp at 42 kDa (Fig. 1B). Incubation with phospho-(Ser/Thr) PKA substrate antibodies (Fig. 3A and B) revealed that 7 proteins changed their phosphorylation state after motility activation in spermatozoa of sterlet. While the 95 kDa protein appears to be phosphorylated only in immotile sperm, the degree of phosphorylation increased following activation for the 6 other proteins (90, 57, 55, 43, 38 and 30 kDa bands). The phosphorylation of the 57, 55 and 43 kDa proteins was sensitive to H89 inhibition. A different pattern of phosphorylation was observed for carp spermatozoa, for which 9 proteins changed their phosphorylation state. The 125, 49, 30 and 27 kDa proteins were modified in a transient manner, with phosphorylation increasing at the beginning of the motility period and disappearing after 30 s of motility, suggesting a role for these phospho-proteins in motility activation rather than in its maintenance. The phosphorylation of the 90, 79, 62 and 45 kDa proteins was maximal in immotile sperm and dropped significantly following activation. Finally, a 40 kDa protein appeared phosphorylated only in immotile spermatozoa. Incubation of immotile carp spermatozoa with the inhibitor H89 led to dephosphorylation of almost all bands except the 79 kDa protein.

Localization of phospho-(Ser/Thr) PKA substrates using immunolabeling and fluorescence microscopy showed that in sterlet, PKA substrates were concentrated in flagella and heads of immotile spermatozoa (Fig. 3C). 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 (Fig. 3D). Interestingly, results of immunolabeling in carp spermatozoa show increase in the level of protein phosphorylation on the spermatozoon flagella after motility activation, although this was not

Table 1 The effect of kinase inhibitors on spermatozoa of sterlet (Acipenser ruthenus) and carp (Cyprinus carpio L.).

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Function</th>
<th>Tested concentrations</th>
<th>Sterlet motility (%)</th>
<th>Carp motility (%)</th>
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<tbody>
<tr>
<td>Control (DMSO)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Staurosporine</td>
<td>Broad spectrum inhibitor, ATP-site competitor</td>
<td>200 μM</td>
<td>80.2 ± 3.62 A</td>
<td>79.5 ± 7.054 A</td>
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<tr>
<td></td>
<td></td>
<td>100 μM</td>
<td>7.9 ± 1.165 D</td>
<td>66.6 ± 9.67 A</td>
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<tr>
<td></td>
<td></td>
<td>50 μM</td>
<td>67.8 ± 5.034 B</td>
<td>68.6 ± 4.074 A</td>
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<tr>
<td></td>
<td></td>
<td>10 μM</td>
<td>72.6 ± 8.37 A</td>
<td>71.9 ± 9.575 A</td>
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<td>Tyrhostin 23</td>
<td>Receptor type protein tyrosine kinase (PTK)</td>
<td>100 μM</td>
<td>76.9 ± 0.29 A</td>
<td>68.5 ± 9.703 A</td>
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<td></td>
<td>50 μM</td>
<td>78.1 ± 5.68 A</td>
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<td></td>
<td>10 μM</td>
<td>80.5 ± 6.47 A</td>
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<td>Go 6983</td>
<td>Ca2+-dependent PKC α1 and β1</td>
<td>100 μM</td>
<td>73.5 ± 0.39 A</td>
<td>68.5 ± 9.192 A</td>
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<td></td>
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<td>50 μM</td>
<td>77.8 ± 7.94 A</td>
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<td></td>
<td>10 μM</td>
<td>79.1 ± 6.94 A</td>
<td>70.8 ± 5.89 A</td>
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<tr>
<td>Chelerythrine</td>
<td>PKC inhibitor</td>
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<td>100 μM</td>
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<td>H-89</td>
<td>ATP-site competitor for PKA</td>
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<td>100 μM</td>
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<td>10 μM</td>
<td>70.3 ± 4.074 A</td>
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Data represent mean values ± s.e. Different letters indicate significant differences within a column (Kruskal–Wallis test, P<0.05).
observed with Western blots, which could be attributed to insolubility of some proteins in extraction medium.

In immotile sterlet spermatozoa, PKA was localized in the flagella of a spermatozoon (Fig. 1D), like the PKA substrates (Fig. 3C). In carp spermatozoa PKA was localized in the midpiece of a spermatozoon (Fig. 1D), similarly to phosphorylated PKA substrates (Fig. 3D).

### Protein phosphorylation on serine residue

In both species, the phosphorylation pattern on three amino acid residues (serine, threonine and tyrosine) changed after motility activation (Figs 4, 5 and 6). Incubation with phosphoserine antibodies revealed 11 protein bands, which increased their phosphorylation state in spermatozoa of sterlet (Fig. 4A) (molecular weights of 98, 80, 62, 58, 55, 49, 45, 38, 30, 25 and 20 kDa). In carp spermatozoa (Fig. 4B), 10 proteins were found to be phosphorylated (125, 70, 60, 45, 43, 30 and 15 kDa) or dephosphorylated (150, 79, 49 kDa) after motility activation.

Proteins with phosphorylated serine residue were observed in spermatozoa flagella and heads in both species (Fig. 4C and D). In carp spermatozoa, motility activation led to enhanced serine phosphorylation at the head of spermatozoa.

### Protein phosphorylation on tyrosine residue

The Western blot analysis with antibodies to phosphotyrosine residues revealed that in sterlet spermatozoa, 6 proteins (of 120, 65, 55, 45, 25 and 20 kDa) appeared to increase in phosphorylation 4 s after motility activation and then were dephosphorylated by the end of motility period (Fig. 5A). Three protein bands (of 58, 48 and 39 kDa) were gradually dephosphorylated after motility activation compared to control (Fig. 5A). A total of 6 proteins were phosphorylated (65, 59, 45, and 35 kDa) or temporarily modified (90, 25 kDa) in carp spermatozoa (Fig. 5B).

Immunolocalisation of phosphotyrosine residue showed that in immotile sterlet spermatozoa, tyrosine phosphorylation occurred in a gradient manner along the flagellum, the signal disappearing toward the tip of the tail (Fig. 5C). In activated sperm, the fluorescent signal decreased in the flagellum but
increased in the head. In carp spermatozoa, however, tyrosine phosphorylation could be seen along the flagellum after motility activation, and in immotile spermatozoa, the majority of phosphorylated proteins were localized in the head of the spermatozoon (Fig. 5D).

Protein phosphorylation on threonine residue
In spermatozoa of sterlet, 3 proteins of 59, 51 and 42 kDa increased phosphorylation on threonine residue up to 30 s after motility activation with subsequent dephosphorylation at the end of motility period.
In carp sperm, motility activation led to the dephosphorylation of a 56 kDa protein (Fig. 6B) while three proteins of 60, 45 and 27 kDa were modified in a transient fashion like those in sterlet sperm (Fig. 6A).

(Fig. 6A). In carp sperm, motility activation led to the dephosphorylation of a 56 kDa protein (Fig. 6B) while three proteins of 60, 45 and 27 kDa were modified in a transient fashion like those in sterlet sperm (Fig. 6A).

Overall, serine and threonine phosphorylation (Figs 3A and B and 6A and B) did not completely correspond...
Protein phosphorylation in fish sperm motility

Figure 4 Reaction of anti-phosphoserine antibody with spermatozoa from sterlet (A and C) or carp (B and D). (A and B) Western blots on proteins extracted from spermatozoa either in immobilizing medium (control, C) or at different times (in seconds) after motility activation. Arrows on the right indicate protein bands that changed phosphorylation state after motility activation: $i =$ increased phosphorylation, $d =$ decreased phosphorylation. S numbers correspond to spots which were identified by mass spectrometry and are listed in Table 2 (sterlet) or Table 3 (carp). Molecular weight markers (kDa) are on the left. Relative intensities of phosphorylated bands are summarized in tables below the Western blot images. Values are expressed as means $\pm$ S.E. ($n = 3$). Small letters (a, b, c) indicate significant differences within a row (Kruskal–Wallis test, $P < 0.05$). (C and D) Immunolocalization of proteins phosphorylated on serine residue in immotile and activated spermatozoa which were attached to glass slides and fixed: $h =$ head of spermatozoon, $fl =$ flagella. Bars represent approximately 10 $\mu$m.

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Figure 5 Reaction of anti-phosphotyrosine antibody with spermatozoa from sterlet (A and C) or carp (B and D). (A and B) Western blots on proteins extracted from spermatozoa either in immobilizing medium (control, C) or at different times (in seconds) after motility activation. Arrows on the right indicate protein bands that changed phosphorylation state after motility activation: i = increased phosphorylation, d = decreased phosphorylation, t = temporarily modified band. S numbers correspond to spots which were identified by mass spectrometry and are listed in Table 2 (sterlet) or Table 3 (carp). Molecular weight markers (kDa) are on the left. Relative intensities of phosphorylated bands are summarized in tables below the Western blot images. Values are expressed as means ± s.e. (n = 3). Small letters (a, b, c) indicate significant differences within a row (Kruskal–Wallis test, P < 0.05). (C and D) Immunolocalization of proteins phosphorylated on tyrosine residue in immotile and activated spermatozoa which were attached to glass slides and fixed: h = head of spermatozoon, fl = flagella. Bars represent approximately 10 µm.
Protein phosphorylation in fish sperm motility

Figure 6 Reaction of anti-phosphothreonine antibody with spermatozoa from sterlet (A and C) or carp (B and D). (A and B) Western blots on proteins extracted from spermatozoa either in immobilizing medium (control, C) or at different times (in seconds) after motility activation. Arrows on the right indicate protein bands that changed phosphorylation state after motility activation: d = decreased phosphorylation, t = temporarily modified band. S numbers correspond to spots which were identified by mass spectrometry and are listed in Table 2 (sterlet) or Table 3 (carp). Molecular weight markers (kDa) are on the left. Relative intensities of phosphorylated bands are summarized in tables below the Western blot images. Values are expressed as means ± s.e. (n = 3). Small letters (a, b, c) indicate significant differences within a row (Kruskal–Wallis test, P < 0.05). (C and D) Immunolocalization of proteins phosphorylated on threonine residue in immotile and activated spermatozoa which were attached to glass slides and fixed: h = head of spermatozoon, fl = flagella, mp = midpiece. Bars represent approximately 10 µm.
to phosphorylation of PKA substrates in spermatozoa of carp and sterlet (Fig. 3A and B). This fact indicates higher sensitivity of phospho-PKA substrate antibodies compared to less specific antibodies to phosphorylated residues, as has been recently reported for reptile spermatozoa (Nixon et al. 2016).

Incubation with anti-phosphothreonine antibodies revealed a weak phosphorylation of flagella in sterlet spermatozoa. Most phosphorylated proteins were localized in the head of immotile spermatozoa and in the midpiece of motile spermatozoa (Fig. 6C). In carp, phosphorylation on threonine residue occurred mainly in the head and midpiece of immotile spermatozoa and translocated to the principal part of flagella after motility activation. (Fig. 6D).

**Identification of proteins whose phosphorylation state is modified following motility activation**

To identify phosho-proteins involved in spermatozoa motility, proteins extracted from spermatozoa before and after motility activation were separated with 2DE, blotted on PVDF membrane and incubated with antibodies to either phospho-(Ser/Thr) PKA substrate, phospho-(Ser) PKC substrate, phosphoserine, phosphotyrosine or phosphothreonine (Supplementary Fig. 2A, B, C, D and E, respectively). Figure 7 represents total protein profiles (Coomassie Brilliant Blue R-250 stain) of sterlet and carp spermatozoa before 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 substrates, phosphorylated or dephosphorylated after motility activation (Supplementary Fig. 2A). Spot 6 in sterlet spermatozoa was found to be a phospho-(Ser) PKC substrate, slightly dephosphorylated after motility activation (Supplementary Fig. 2B). Incubation with anti-phosphoserine antibodies revealed an increased phosphorylation of spots 7, 12, 15–18 in sterlet spermatozoa and of spots 7–13 in carp after motility activation (Supplementary Fig. 2C). Spot 5 in carp spermatozoa was dephosphorylated on serine residue after motility activation. Spots 5, 7–9, 11, 13 and 14 in sterlet spermatozoa were dephosphorylated on tyrosine residue after motility activation, whereas spots 10 and 12 in sterlet sperm and spots 3, 12, 14–16 in carp were phosphorylated on tyrosine during motility period (Supplementary Fig. 2D). Protein phosphorylation on threonine residue was weak in spermatozoa of both species. Only spot 2 in carp sperm was identified with anti-phosphothreonine antibody (Supplementary Fig. 2E).

Proteins of interest were cut from 2D gels (Fig. 7) and analyzed 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 phosphorylated during sperm motility is provided in Tables 2 and 3, respectively, together with their accession number, molecular mass, sequence coverage, number of unique peptides, phosphorylated amino acid residue and the main biological function. The majority...
<table>
<thead>
<tr>
<th>Spot no.</th>
<th>Phosphorylated amino acid</th>
<th>Protein name (organism)</th>
<th>Accession no.</th>
<th>Calculated MW (kDa)</th>
<th>Observed MW (kDa)</th>
<th>pI</th>
<th>MASCOT score</th>
<th>Sequence coverage (%)</th>
<th>Number of peptides (ion score ≥ 30)</th>
<th>Protein function</th>
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<td>Spot 1</td>
<td>Serine/Threonine (PKA substrate)</td>
<td>PREDICTED: creatine kinase S-type, mitochondrial-like isoform X2 (Neolamprologus brichardi)</td>
<td>gi</td>
<td>584004697</td>
<td>ref</td>
<td>XP_006797349.1</td>
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<td>8.04</td>
<td>43.9</td>
<td>7.9</td>
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<td>Spot 2</td>
<td>Serine/Threonine (PKA substrate)</td>
<td>Malate dehydrogenase, mitochondrial (Danio rerio)</td>
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<td>47085883</td>
<td>ref</td>
<td>NP_998296.1</td>
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<td>Phosphoglycerate kinase, partial (Acipenser baerii)</td>
<td>gi</td>
<td>46849425</td>
<td>dbj</td>
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<td>Serine (PKC substrate)</td>
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<td>573876979</td>
<td>ref</td>
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<td>gb</td>
<td>ABF60009.1</td>
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<td>213512628</td>
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<td>432883664</td>
<td>ref</td>
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<td>Septin-7-like isoform X3 (Stegastes partitus)</td>
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<td>657584138</td>
<td>ref</td>
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<td>Spot 12</td>
<td>Tyrrosine, serine</td>
<td>Triosephosphate isomerase (Acipenser brevirostrum)</td>
<td>gi</td>
<td>15149246</td>
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<td>AF387818_1</td>
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<td>PREDICTED: phosphoglucomutase-1-like isoform X2 (lepisosteus oculatus)</td>
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<td>573894328</td>
<td>ref</td>
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<td>61.970</td>
<td>5.94</td>
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(Continued)
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 each of these findings and their potential meaning for fish sperm motility.

Discussion

After spawning into an aqueous medium, 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. After reception of extracellular signals by specific ion channels or receptors in the spermatozoon, intracellular signals are transferred to the sperm axoneme through protein phosphorylation, Ca2+ signaling and cAMP-dependent pathways (Inaba 2003). For the two evolutionarily distant fish species, common carp (Cyprinus carpio L.) and sturgeon (Acipenser ruthenus), not only does the trigger of motility activation differ (Bondarenko et al. 2013), 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.

Involvement of PKA and PKC in sperm motility

Results of motility analyses in the presence of kinase inhibitors showed that PKC participates in motility activation in both species, while PKA seems to be involved in motility only for carp spermatozoa. It should be mentioned that the specificity of inhibitors is never perfect, and that various isoforms of each kinase may be expressed (White et al. 2007). For example, 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. 5 and Supplementary Fig. 2), indicating that they may be involved in initiating or regulating spermatozoa motility. Furthermore, cherythrine, the inhibitor of PKC, has been shown to inhibit only lipid-stimulated and not basal kinase activity (Herbert et al. 1990). Thus, it can be speculated that the specificity of this inhibitor is lower compared to Go 6983 that, however, had no effect on fish sperm motility. Taking these facts into account, we suggest that further studies with a series of inhibitors are needed to evaluate the role of each kinase in fish spermatozoa motility.

Numerous studies have described the role of PKA in sperm motility of different species (Visconti et al. 1995, Inaba 2003, Lasko et al. 2012, Nixon et al. 2016). It is generally assumed that the activity of PKA depends on

Table 2

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<tr>
<th>Spot no.</th>
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<th>Protein name/organism</th>
<th>Protein Accession no.</th>
<th>Calculated MW</th>
<th>Calculated pI</th>
<th>Calculated Score</th>
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<th>Observed pI</th>
<th>Observed Score</th>
<th>Percent Phos.</th>
<th>Number of Peptides</th>
<th>Protein Function</th>
</tr>
</thead>
</table>
| Tyrine   | PREDICTED: phosphorylated leucine-
| Spot 14  | Calmodulin (Oreochromis mossambicus) | gi|78099193|sp|Q6R520.3|CALM_OREMO | 16.835 | 4.05 | 318 | 55 | 6 | Calcium ion binding, intracellular signaling |
| Serine   | Spot 15                  | PREDICTED: phosphoglucomutase-1-like isoform X1 (Lepisosteus oculatus) | gi|573894326|ref|XP_006634913.1 | 62.515 | 5.94 | 501 | 16 | 5 | Carbohydrate metabolic process |
| Serine   | Spot 16                  | PREDICTED: pyruvate kinase PKM-like isoform X2 (Lepisosteus oculatus) | gi|573881641|ref|XP_006629014.1 | 58.556 | 6.18 | 264 | 21 | 3 | ATP biosynthetic process |
| Serine   | Spot 17                  | Creatine kinase (Takifugu rubripes) | gi|8575804 | 46.852 | 7.12 | 39 | 7.2 | 145 | 10 | 3 | Glycerol metabolic process |
| Serine   | Spot 18                  | PREDICTED: l-lactate dehydrogenase A chain-like (Lepisosteus oculatus) | gi|573909562|ref|XP_006642499.1 | 36.934 | 7.12 | 145 | 10 | 3 | Glycolytic process, NAD metabolic process |
Table 3  Carp sperm phospho-protein identification by MALDI-TOF-MS/MS.

<table>
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<tr>
<th>Phosphorylated amino acid</th>
<th>Protein name (organism)</th>
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<th>pl</th>
<th>Observed MW (kDa)</th>
<th>pl</th>
<th>MASCOT score</th>
<th>Sequence coverage (%)</th>
<th>Number of peptides (ion score ≥30)</th>
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<td>Serine/Threonine (PKA substrate)</td>
<td>PREDICTED: isocitrate dehydrogenase (NADP); mitochondrial-like (Poecilia formosa)</td>
<td>gi[617389834]</td>
<td>ref</td>
<td>XP_007549128.1</td>
<td>51.485</td>
<td>7.53</td>
<td>50</td>
<td>7.5</td>
<td>226</td>
<td>14</td>
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<td>Serine/Threonine (PKA substrate), Threonine</td>
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<td>gi[658914147]</td>
<td>ref</td>
<td>XP_008396965.1</td>
<td>43.718</td>
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<td>43.6</td>
<td>7.8</td>
<td>368</td>
<td>21</td>
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<td>Tyrosine, Serine/Threonine (PKA substrate)</td>
<td>Brain creatine kinase (Danio rerio)</td>
<td>gi[116004537]</td>
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<td>NP_001070631.1</td>
<td>43.124</td>
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<td>Serine</td>
<td>Parvalbumin-7-like (Cynoglossus semilaevis)</td>
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<td>XP_008328183.1</td>
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(Continued)
the concentration of cAMP, which is in turn regulated by adenylyl cyclase and phosphodiesterase. The binding of cAMP to PKA regulatory subunits promotes the dissociation of the active catalytic subunits, which can then phosphorylate target proteins on serine/threonine residues (Urner & Sakkas 2003). Involvement of PKA in fish spermatozoa 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, we show both by Western blotting and immunolabeling that sterlet and carp spermatozoa harbor PKA and that proteins are phosphorylated by PKA differently before and after sperm motility activation. Moreover, the phosphorylation patterns differ between sterlet and carp over time after activation, with a global rise in phosphorylation for sterlet protein extract compared to a decrease of global phosphorylation in carp extracts (Fig. 3A and B). This may reflect a species difference in the requirement of cAMP-activated kinase for motility activation vs motility maintenance. We found that 4 proteins (125, 49, 30 and 27 kDa) were transiently phosphorylated between 4 and 30 s following sperm activation in carp. Two of these proteins were identified as isocitrate dehydrogenase (NADP), mitochondrial-like (51 kDa) and adenylate kinase isoenzyme 1 (20 kDa) involved in energy production pathways (Table 3). It is known that the activation of motility does not depend on cAMP in carp (Cosson & Gagnon 1988, Krasznai et al. 2000); therefore, these proteins are probably involved in the phase of motility maintenance. This result is in agreement with the recently published study of Dzyuba and coworkers that reported adenylate kinase activity in motile spermatozoa of carp and sterlet (Dzyuba et al. 2016).

Localization of PKA and PKA substrates and results of protein identification in carp spermatozoa (Figs 1D and 3D) supports the hypothesis that PKA is involved in energy production and motility maintenance. Thus, 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 3). All these enzymes are involved in ATP metabolism and play important roles in cellular energy homeostasis.
In sterlet, results of Western blotting and immunolabeling seem to indicate involvement of PKA in sperm motility. That was expected, since sturgeon spermatozoa motility activation is cAMP dependent (Linhart et al. 2002). However, in sterlet, the PKA inhibitor H-89 had only a slight effect on sperm motility at a concentration far above that recommended for specificity. Incubation with H-89 led to a decrease in PKA substrate phosphorylation in both carp and sterlet spermatozoa, indicating specificity of the inhibitor. Thus, observed phosphorylation of PKA substrates in sterlet spermatozoa seems to be involved in maintenance of motility rather than in its activation.

In both quiescent and motile sterlet spermatozoa, PKA and PKA substrates were localized in the head and flagella (Figs 1D and 3C). The PKA substrates we identified were mitochondrial proteins: creatine kinase S-type and malate dehydrogenase and cytoplasmic proteins: phosphoglycerate kinase, glyceraldehyde-3-phosphate dehydrogenase and phosphoglycerate mutase. These 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 the glycolytic pathway. Several studies have previously described the role of PKA in mitochondria function in muscle and liver (Lark et al. 2015), and in bovine spermatozoa (Mizrahi & Breitbart 2014). Hence, it is possible to speculate that PKA activity regulates ATP generation and/or transport along the flagella in order to sustain spermatozoa motility in carp and sterlet.

PKC has been 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). Previous studies reported the involvement of PKC in the acrosome reaction and gamete fusion (reviewed by Breitbart & Naor 1999); however, less is known about the function of PKC in sperm motility activation. In the current study, motility was blocked in the presence of the PKC inhibitor (chelerythrine) both in C. carpio and A. ruthenus spermatozoa, and results of Western blotting and immunolabeling indicated the presence of PKC and phosphorylated PKC substrates in spermatozoa of both species. Though we were not able to identify PKC substrates in carp spermatozoa due to low signal from antibodies on immunoblots of 2D gels, the involvement of protein kinase C and casein kinase substrate protein in carp sperm motility has been recently reported (Dietrich et al. 2016). Thus, our results indicate that this protein is temporally phosphorylated during motility period. The PKC substrate in sterlet spermatozoa was identified as dynein intermediate chain 2 (IC2). This protein was phosphorylated in immotile spermatozoon and its dephosphorylation in the presence of chelerythrine correlated with a blocking of motility.

Identified phosho-proteins involved in sperm motility

We observed that many proteins changed their phosphorylation status after motility activation (Figs 4, 5 and 6). Unfortunately, we were not able to identify all phosho-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 the glycolytic pathway and regulate energy production. Results of immuno-fluorescence labeling indicate that phosphoserine proteins are localized in the head and along the flagellum of sterlet spermatozoa. Increased intensity of fluorescence after motility activation (Fig. 4C) correlates with results of Western blotting and may reflect the 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 s in carp), as well as the slow

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ATP decrease in sterlet spermatozoa during the motility period (Billard et al. 1999, Dzyuba et al. 2016).

For comparison, among the 9 serine-phosphorylated carp sperm proteins we identified, only 3 proteins belong to energy metabolic pathways (creatine kinase B-type, l-lactate dehydrogenase B-A chain, and malate dehydrogenase). The rest of the serine-phospho-proteins are axonemal (septin-8-A, parkin coregulated gene protein homolog), calcium-binding (parvalbumin-7-like, parvalbumin 6) or heat shock (Hsp90 and Hsp70) proteins. Thus, compared to sterlet spermatozoa, in carp only a few proteins are involved in energy production during motility period. Therefore, it can be suggested that carp spermatozoa motility mostly depend on ATP produced and stored prior to motility activation.

In both species of fish, some of the tyrosine-phosphorylated proteins belong to ATP production and energy homeostasis pathways as well: 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. As was the case for serine-phospho-proteins, the most abundant tyrosine-phospho-proteins in sterlet spermatozoa were proteins associated with glycolysis and energy metabolism. Results of Western blotting indicate increased tyrosine phosphorylation right after motility activation and slow dephosphorylation of tyrosine residues in proteins up to the end of motility period, which correlates well with temporal requirements for energy production.

Our results suggest that in addition to mitochondria functions and glycolysis other signaling pathways are also regulated by protein phosphorylation. Thus, two heat shock proteins (HSPs) were phosphorylated after motility activation in carp spermatozoa. The role of HSPs in spermatozoa has been studied in mammals (Cole & 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 (Hsp70) and heat shock protein 90 (Hsp90) 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 HSP70 under osmotic stress (Cole & Meyers 2011), and of HSP90 during human sperm capacitation (Li et al. 2014). HSP70 was shown to localize in the head and HSP90 in the midpiece of monkey spermatozoa (Cole & Meyers 2011). This corresponds to our immunolabeling results, which showed an increased phosphorylation on serine residues in the head and midpiece of carp spermatozoa after motility activation.

Our study revealed that calmodulin (CaM) is regulated by tyrosine phosphorylation in sterlet spermatozoa. Involvement of CaM in sperm motility has been reported for many other aquatic species, such as tilapia (Morita et al. 2006) and ascidians (Nomura et al. 2004). CaM is considered to be a ubiquitous protein mediating intracellular Ca2+ signaling, which activates sperm motility through the activation of dynein ATPase (Tash et al. 1988). Hence, our results are in agreement with previous studies, which showed Ca2+ dependence of sturgeon sperm motility activation (Alavi et al. 2011). In spermatozoa of carp, we found serine phosphorylation on parvalbumin (PV), a calcium-binding protein with three EF-hand motifs, which is structurally related to CaM. A previous study reported the presence of PV and CaM in carp spermatozoa and their participation in sperm motility (Dietrich et al. 2016). Results of the current study indicate that activity of both proteins is controlled by phosphorylation. Interestingly, compared to other Ca2+-binding proteins, such as CaM, PV has a slow dissociation and association rate, due to the fact that Mg2+ ions compete with Ca2+ ions for binding (Caillard et al. 2000). Hence, it is possible that the presence of PV in carp spermatozoa allows a different Ca2+-signaling mechanism, compared to spermatozoa of species which mostly rely on CaM such as sterlet (Table 2) or tilapia (Morita et al. 2006). Further studies are needed to understand the role of PV in spermatozoa of common carp.

In a previous study, we found that septin-8-A is dephosphorylated during 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 (Supplementary Fig. 2). We also detected septin 7 as a protein phosphorylated on tyrosine in sterlet sperm. Septins are a highly conserved family of GTP-binding proteins that co-localize with cellular membranes and the microtubule cytoskeleton (Spiliotis 2010). Studies on mammals showed that septins are localized in the annulus – a fibrous structure between the 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 yet clear. 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 idea is supported by increased alpha-tubulin phosphorylation in carp spermatozoa (Fig. 5B, Supplementary Fig. 2, and Table 3), where septin-8-A was also dephosphorylated after motility activation. Immunofluorescent localization of phosphorytrosine 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.
Flotillin-1 was identified in spermatozoa of both species as being 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 spermatozoon induced redistribution of flotillin-1 within the plasma membrane of the spermatozoon head (van Gestel et al. 2005). Moreover, tyrosine phosphorylation is known to control the distribution of flotillin microdomains (Riento et al. 2009), and sperm capacitation in different species (Aitken & Nixon 2013, Nixon et al. 2016). In our study, tyrosine phosphorylation of the head membrane 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. To our knowledge, this is the first evidence of capacitation-like processes in fish spermatozoa.

**Conclusion**

In conclusion, spermatozoa 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 the two species. Thus, PKA seems to play an important role in energy production and the sustaining of carp and sterlet spermatozoa motility. However, despite the fact that carp spermatozoa movement mainly depends on ATP stored before activation (Perchec et al. 1995), inhibition of PKA activity led to a significant reduction in percentage motile cells. On the other hand, sterlet spermatozoa movement was less sensitive to PKA inhibition. This could be due to the abundance of glycolytic enzymes in sterlet spermatozoa, which may indicate the presence of other, PKA-independent, pathways of ATP production.

Furthermore, the difference in phospho-(Ser) PKC substrate abundance between carp and sterlet spermatozoa, as well as the presence of PV instead of CaM in carp sperm, may reflect different types of involvement of Ca²⁺ signaling in sperm motility. Further studies are needed to understand the mechanisms of Ca²⁺ 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 after motility activation may reflect an activation of signaling pathways involved in gamete fusion and fertilization.

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.

**Supplementary data**

This is linked to the online version of the paper at http://dx.doi.org/10.1530/REP-16-0662.

**Declaration of interest**

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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