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Involvement of redox- and phosphorylation-dependent pathways in osmotic adaptation in sperm cells of euryhaline tilapia

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SUMMARY

Sperm cells involved in fertilisation must tolerate hypo-osmotic and hyper-osmotic environments. Euryhaline tilapia (*Oreochromis mossambicus*) can acclimatise to and reproduce in freshwater and seawater because its sperm are able to adapt to these differing osmotic environments. In this study, we found that the dephosphorylation of sperm proteins in *O. mossambicus* correlated with the activation of flagellar motility when sperm were exposed to hypotonic or hypertonic conditions, and that differences in phosphorylation may reflect adaptations to a given osmotic environment. Of the sperm proteins that were dephosphorylated, the phosphorylation pattern of an 18 kDa protein, identified as the superoxide anion scavenger Cu/Zn superoxide dismutase (Cu/Zn SOD), was different in freshwater- and seawater-acclimatised tilapia sperm. Cu/Zn SOD was distributed from the sperm head to the flagellum. Additionally, differences were observed between freshwater and seawater tilapia in the nitration of tyrosine residues (which might be mediated by SOD) in sperm flagellar proteins in response to osmotic shock. These results demonstrate that reactive-oxygen-species-dependent mechanisms contribute to both osmotic tolerance and the activation of flagellar motility.

INTRODUCTION

Euryhaline tilapia (*Oreochromis mossambicus*) is a good model for studying cellular mechanisms of adaptation to osmotic environments because it spawns in both freshwater and seawater (Brock, 1954). The sperm of *O. mossambicus* acclimatise to their habitat *via* acclimation of the fish itself (Linhart et al., 1999; Morita et al., 2004). They must reach unfertilised eggs by means of flagellar motility, and need to maintain motility in a wide range of osmotic environments. Modulation of spermatogenesis occurs during acclimatisation of the fish to different osmotic environments. In the gill epithelium, cilia also adapt to changes in ionic strength and osmotic pressure (Laurent and Dunel, 1980). This adaptation involves modulation of ATPase and ion transporter activity, and changes in structural proteins (Evans, 2002). The adaptation response is based on the turnover of gill cilia and altered gill cell differentiation, which occur as a result of altered transcription (Tipsmark et al., 2002; Fiol and Kultz, 2005; Fiol et al., 2006). The architecture of the sperm flagellum is similar to that of the cilium, and the motility apparatuses of the flagellum and the cilium are structurally identical (Gibbons, 1981; Inaba, 2003). Transcriptional changes similar to those in cilia may occur in sperm flagella during spermatogenesis for them to be able to maintain motility in different osmotic environments. It should be noted, however, that the roles of cilia and flagella differ: whereas the former play many roles in cell signalling and in the maintenance of cellular homeostasis (reviewed by Bisgrove and Yost, 2006), the latter act to propel sperm to unfertilised eggs. The available evidence suggests that flagella may have evolved from cilia and subsequently developed specific features (Mitchell, 2007). Based on previous studies, it seems reasonable that signals regulating motility and acclimatisation of in these two organelles may differ. Thus, it seems likely that the osmotic adaptation of sperm flagella is regulated by mechanisms distinct from those that control this process in cilia.

Sperm flagellar motility is regulated by osmotic stress (Morisawa, 1994). Indeed, the stimuli for its activation differ between freshwater and marine teleosts: hypotonic shock activates motility in the sperm of freshwater teleosts, whereas hypertonic shock produces a similar effect in the sperm of marine teleosts (Morisawa and Suzuki, 1980; Oda and Morisawa, 1993). These osmolality-dependent responses are thus specialised to the different habitats. Differences in osmolality between the internal and external environments activate intracellular signals.

Responses to osmotic pressure by the sperm of freshwater-acclimatised tilapia (FWT) and seawater-acclimatised tilapia (SWT) are also different (Linhart et al., 1999; Morita et al., 2004). Additionally, osmotic regulation appears to differ in FWT and SWT sperm. SWT sperm retain their sleeve structure at the neck region of the sperm head, even when they are exposed to a high-osmolality environment (Morita et al., 2004). In contrast, the sleeve structure of FWT sperm shrinks under such conditions (Morita et al., 2003). Moreover, the signalling pathway by which Ca^{2+} mobilisation and, ultimately, motility occur is also different in FWT and SWT sperm (Morita et al., 2003; Morita et al., 2004). This might be a response to the Ca^{2+} concentration in the external environment, with high-osmolality environments containing larger quantities of Ca^{2+} than seawater. Osmotic regulation and the modulation of Ca^{2+} mobilisation occur at the plasma membrane. Presumably, alterations in the receptors detecting osmotic stimuli occur at the plasma membrane.

In this study, we focused on differences in the patterns of sperm protein phosphorylation in FWT and SWT sperm subjected to osmotic stress. We found that several proteins had different patterns of phosphorylation in FWT and SWT sperm, one of which was identified as a Cu/Zn superoxide dismutase (SOD1). We explored the potential role of SOD1 in the regulation of sperm motility by

investigating redox-dependent protein modification and its implications for osmotic stress resistance and the regulation of flagellar motility.

MATERIALS AND METHODS

Chemicals

Ampholine, agarose, protein A Sepharose CL-4B, HiTrap NHS-activated HP columns and enhanced chemiluminescence (ECL) chemicals were purchased from GE Healthcare (Little Chalfont, Buckinghamshire, UK). ^{32}P ($H_3{}^{32}PO_4$ in H_2O; $500\,mCi\,ml^{-1}$) was from MP Biomedicals (Irvine, USA). Protein markers and polyvinylidene fluoride (PVDF) membranes were from Bio-Rad Laboratories (San Diego, CA, USA). CHAPS was from Dojindo (Kumamoto, Japan). Anti-Cu/Zn superoxide dismutase polyclonal antibody was from StressGen Biotechnologies (Victoria, BC, Canada) and anti-nitrotyrosine polyclonal antibody was from Upstate Biotechnology (New York, NY, USA). All other chemicals used were reagent grade.

Fish and sperm collection

All tilapia [*Oreochromis mossambicus* (Peters 1958)] used in this study were collected from a brackish-water region of the Aja River located south of Okinawa, Japan. The fishes were then acclimated to freshwater or seawater. The fish were kept in 1000 l freshwater or seawater tanks for at least 1 month before experiments (Morita et al., 2003; Morita et al., 2004). Sperm collections were conducted by adopting the experimental protocol of Morita et al. (Morita et al., 2003). Sperm that were used in this study showed more than 90% motility.

Phosphorylation assay *in vivo*

One volume of isolated sperm was suspended in four volumes of artificial seminal plasma as described by Morita et al. (Morita et al., 2003). $H_3{}^{32}PO_4$ was added to the suspension to a final concentration of $15\,MBq\,ml^{-1}$. The suspension was stored on ice for 5 h. Five volumes of activating solutions [artificial seminal plasmas (ASPs); hypotonic solution ($50\,mmol\,l^{-1}$ NaCl, $5\,mmol\,l^{-1}$ $CaCl_2$ and $10\,mmol\,l^{-1}$ HEPES-NaOH; pH 8.0) or hypertonic solution ($300\,mmol\,l^{-1}$ NaCl, $10\,mmol\,l^{-1}$ $CaCl_2$ and $10\,mmol\,l^{-1}$ HEPES-NaOH; pH 8.0)] was added to the one volume of the $H_3{}^{32}PO_4$-incubated suspensions, which were stored for 5 min at room temperature to induce protein phosphorylations and the accompanying motility activation. Sperm suspensions were centrifuged at 10,000 ***g*** for 5 min at 4°C to obtain pelleted sperm. Extraction solution [$8\,mol\,l^{-1}$ urea, $2\,mol\,l^{-1}$ thiourea, $100\,mmol\,l^{-1}$ dithiothreitol (DTT) and 2% (w/v) CHAPS] was added to pelleted sperm to extract sperm proteins, or Triton X-100 solution ($175\,mmol\,l^{-1}$ potassium acetate, $1\,mmol\,l^{-1}$ EDTA, $1\,mmol\,l^{-1}$ DTT, 0.04% (w/v) NP-40 and $20\,mmol\,l^{-1}$ HEPES-NaOH; pH 8.0) was added to extract the Triton-soluble fraction. Centrifugation at 10,000 ***g*** for 5 min was carried out after extraction to obtain the various fractions. Five times sample buffer was added to each fraction for one-dimensional (1-D) SDS-PAGE. We repeated the assay six times for the 1-D analyses and four times for the 2-D analyses. In each experiment, we used a mixture of semen from two or three males. We did not checked sperm motility after incubation with ^{32}P. We checked sperm motility after incubation for 5 h without $H_3{}^{32}PO_4$ as a pilot study. Sperm motility was reduced by 30–40% compared with before the incubation.

Immunoprecipitation

Immunoprecipitation experiments were carried out according to the method of Satouh et al. (Satouh et al., 2005). Antisera (anti-18-kDa peptide antibody and Cu/Zn superoxide dismutase antibody) were diluted 1:50 with phosphate-buffered saline (PBS), and 1 ml of the solution was mixed with 50 ml of protein-A–Sepharose beads (GE Healthcare). After gentle agitation for 2 h, the beads were washed with PBS repeatedly. Antibody was then immobilised onto the beads with $2.5\,mmol\,l^{-1}$ disuccinimidyl suberate (DSS, Pierce Chemical, Rockford, IL, USA) in PBS for 1 h. The beads were washed with $50\,mmol\,l^{-1}$ glycine, pH 2.5, four times to terminate the cross-linking reaction and to ablate nonspecific binding, followed by equilibration with buffer A ($0.15\,mol\,l^{-1}$ KCl, $20\,mmol\,l^{-1}$ Tris-HCl pH 8.0, $1\,mmol\,l^{-1}$ $MgSO_4$, $0.5\,mmol\,l^{-1}$ EGTA, $0.2\,mmol\,l^{-1}$ DTT). Triton X-100 was added to both beads and membrane fractions to 1%. Both were gently mixed on a rotator at 4°C overnight. The beads were collected by brief centrifugation, and then washed repeatedly with buffer A containing 1% Triton X-100. Proteins were eluted with an equal volume of $100\,mmol\,l^{-1}$ glycine (pH 2.5).

Preparation of antibodies against 18 kDa protein

Polyclonal antibodies against the 18 kDa protein were raised in rabbit. The antigen was a 20 amino acids polypeptide of the 18 kDa protein, which was synthesised using a peptide synthesiser (PPSM 8, Shimadzu, Kyoto, Japan). The synthetic peptide was conjugated with BSA for an antigen. The antigen (200 µg) mixed with complete Freund adjuvant was injected subcutaneously into the rabbit and then 2 weeks later injected again but this time it was mixed with incomplete adjuvant. Antiserum was collected 2 weeks after the second injection. Antibody was purified by affinity chromatography using an antigen-coupled affinity column (Hitrap NHS-activated HP column, 1 ml; GE Healthcare).

Amino acid sequence

Total proteins from the sleeves and flagella were separated by 2-D PAGE and blotted onto a PVDF membrane. Protein spots were cut out from this membrane, and amino acid sequence analyses were carried out using a Peptide sequencer (PPSQ-21, Shimadzu, Kyoto, Japan).

Cross-linkage experiments

The Triton-soluble fraction from FWT sperm was cross linked with 0–$20\,\mu mol\,l^{-1}$ 1-ethyl-3-[3-dimethylaminopropyl]carbodiimide hydrochloride (EDC). EDC ($1\,\mu mol\,l^{-1}$) dissolved in DMSO was added to the Triton-soluble fraction to the appropriate concentrations. Reactions were conducted at room temperature and terminated with 6× sample buffer. Samples were boiled for 2 min at 95°C and kept at –20°C until use.

Electrophoresis

SDS-PAGE (1-D PAGE) was carried out according to Laemmli (Laemmli, 1970). Two-dimensional electrophoresis (2-D PAGE) combined with agarose (GE Healthcare) isoelectric focusing (agarose IEF) was carried out according to the method of Hirabayashi with some modifications (Hirabayashi, 1981). Preblended Ampholine (pH 3.5–9.5) was used as a carrier ampholite. SDS-PAGE in the second dimension was carried out in the same way as described above.

Western blotting analysis

Western blotting was performed according to the methods of Towbin et al. (Towbin et al., 1979) with 10 µg proteins per lane. The resulting SDS-PAGE gels were placed on PVDF membranes and electrically transferred. The membranes were blocked with 5% (w/v) skimmed milk in Tween–TBS [$137\,mmol\,l^{-1}$ NaCl, 0.1%

(w/v) Tween 20 and 20 mmol l^{-1} Tris-HCl; pH 7.4) overnight at 4°C. The membranes were incubated with anti-18-kDa antibody (1/1000), anti-Cu/Zn superoxide dismutase polyclonal antibody (1/2000) or nitrotyrosine (1 μg ml^{-1}). The membranes were washed three times, 10 min each, with Tween–TBS. The membranes were then incubated with HRP-conjugated anti-rabbit IgG (1/5000) in Tween–TBS containing 5% (w/v) skimmed milk for 1 h at room temperature. The membranes were again washed three times and subjected to an ECL reaction, according to the kit protocol, and exposed to X-ray film (Hyper film for ECL; GE Healthcare) for 5–30 s. Protein was measured using the Bradford assay (Bradford, 1976).

Detection of SOD activity

Sleeve and flagellum extracts (20 μg) were applied directly without boiling to a nondenaturing 7.5% polyacrylamide gel. Following electrophoresis, the gel was stained as prescribed (Flohe and Otting, 1984) in a solution containing 50 mmol l^{-1} KH_2PO_4 (pH 7.8), 275 μg ml^{-1} Nitroblue Tetrazolium (NBT), 65 μg ml^{-1} riboflavin and 3.2 μl ml^{-1} TEMED (*N*,*N*,*N*′,*N*′-tetramethylethylenediamine). After a 45 min incubation in the dark, the blue NBT stain for O_2 was developed by exposure to light. Staining was absent at sites of O_2 scavenging (Flohe and Otting, 1984).

Statistical analysis

Intensity of ^{32}P-labelled bands was evaluated with NIH Image software. The mean grey levels in each band were analysed using Fisher's least significant difference (LSD) test. In the analysis of relative band intensities, band intensities of the 18 kDa protein in the ASP was set as the reference and a Dunnet's *post hoc* test was conducted. To detect differences in the 40 kDa band intensities in ASP between FWT and SWT sperm, we evaluated band intensities from five experiments, and a Student's *t*-test was conducted. A probability level of $P<0.05$ was considered significant in all tests. All statistical analyses were conducted in statistical mode in Kaleida Graph v4.0 (HULINKS, Tokyo, Japan).

RESULTS

Protein phosphorylation *in vivo*

The transmission of osmotic stimuli to the motility apparatus is important in initiating sperm flagellar motility (Morisawa, 1994; Alavi and Cosson, 2006). The results of several studies suggest that protein phosphorylation events contribute to motility activation cascades in teleost sperm (Hayashi et al., 1987; Inaba et al., 1999; Morita et al., 2006; Zilli et al., 2008). Thus, osmotic shock and the initiation of flagellar motility through protein phosphorylation seem likely to be linked. In this study, *in vivo* protein phosphorylation

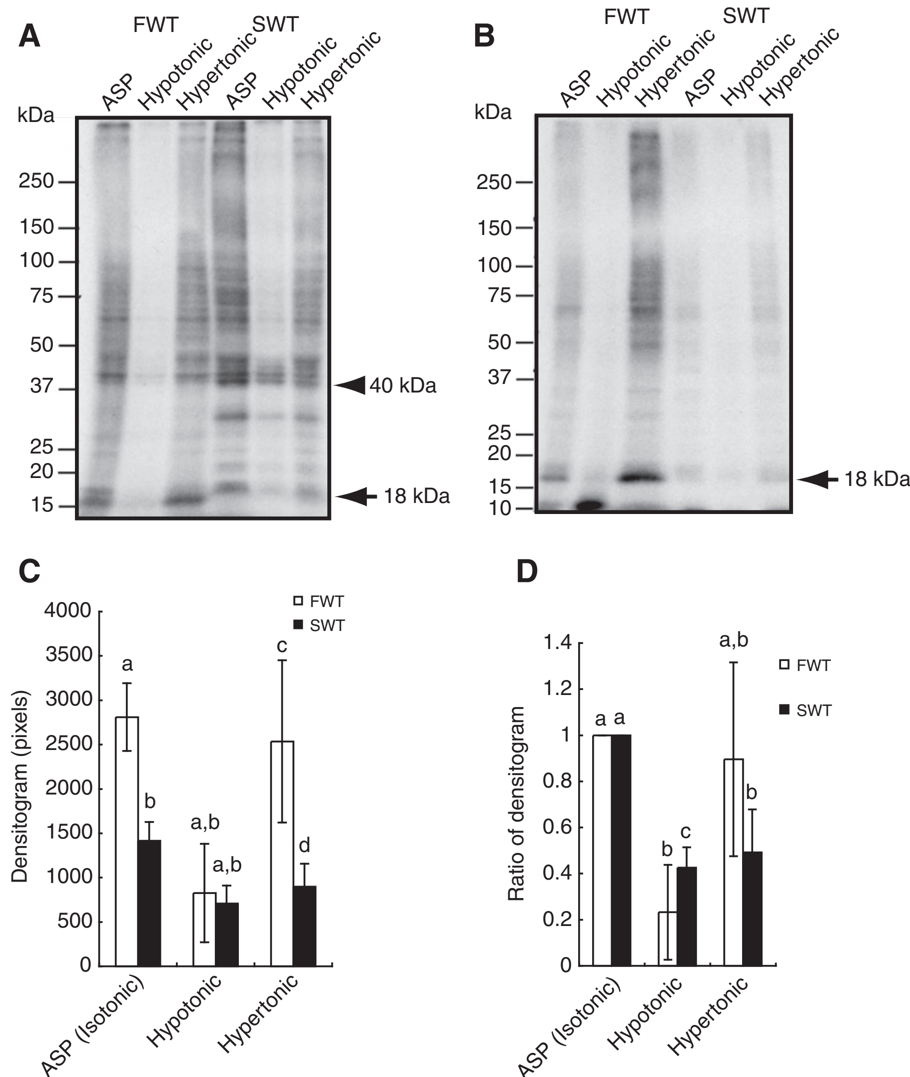

Fig. 1. Effect of osmotic shock on the incorporation of ^{32}P into *O. mossambicus* sperm proteins. Sperm were incubated in artificial seminal plasma (ASP) containing 15 MBq ml^{-1} $H_3{}^{32}PO_4$ for 5 h, and were then suspended in isotonic ASP, a hypotonic solution (50 mmol l^{-1} NaCl, 5 mmol l^{-1} $CaCl_2$) or a hypertonic solution (300 mmol l^{-1} NaCl, 10 mmol l^{-1} $CaCl_2$). All sperm proteins were then separated on a 5–15% gradient gel. (A) Whole-sperm proteins; (B) Triton-soluble fraction; (C) densitogram of the 18 kDa protein; (D) ratio of densitogram of the 18 kDa protein. In C and D, different letters above the bars indicate significant differences (In C, a:b $P<0.01$; c:d $P<0.05$; Fisher's LSD. In D, a:b $P<0.051$; a:c $P<0.005$; Dunnet's *post hoc* test in each salinity; ASP was used as a reference value). FWT, freshwater-acclimatised tilapia; SWT, seawater-acclimatised tilapia; ASP, artificial seminal plasma.

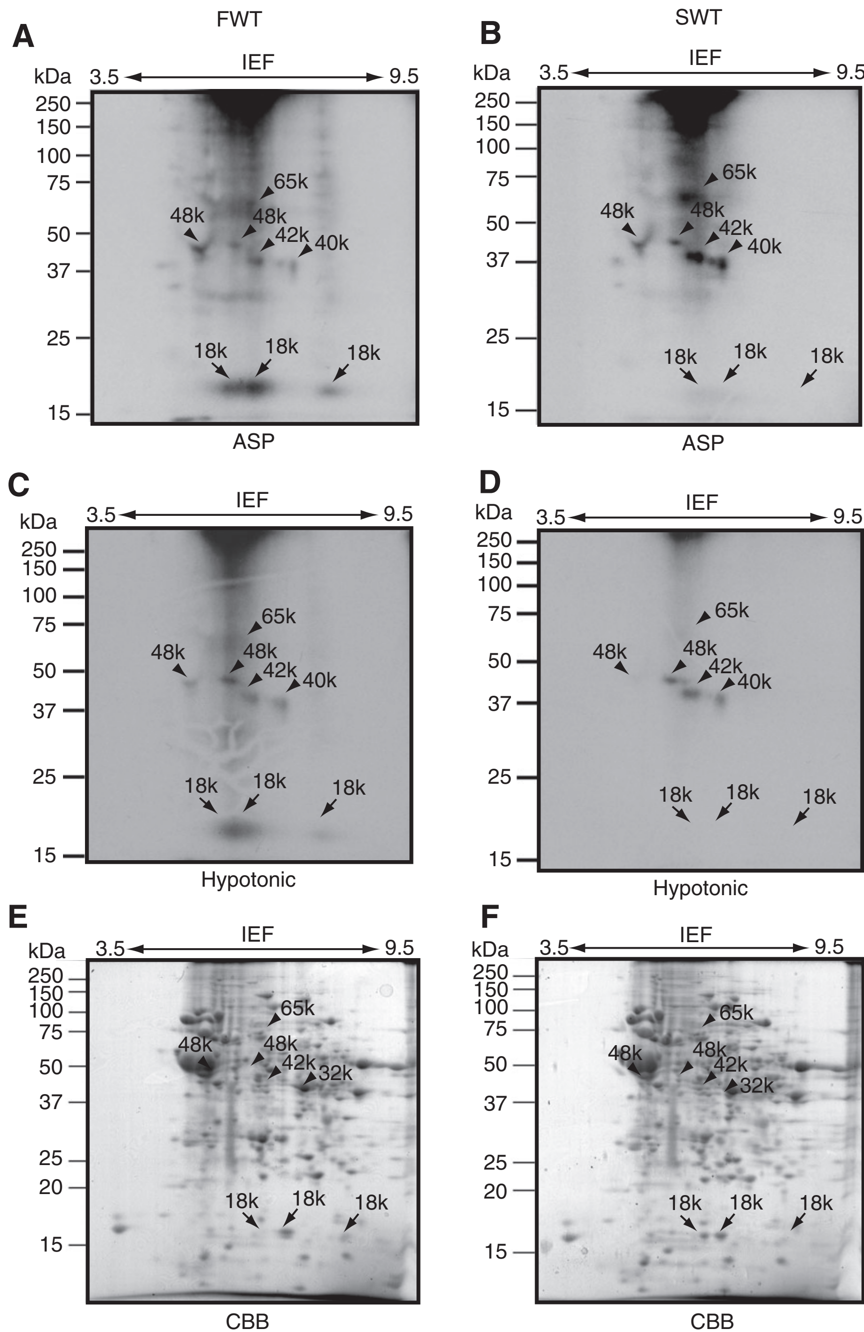

Fig. 2. ^{32}P-labelled proteins separated by two-dimensional SDS–PAGE (using a 12.5% polyacrylamide gel) to elucidate which proteins were labelled with ^{32}P and altered in response to an osmotic shock that induces motility activation. (A,C,E) FWT sperm and (B,D,F) SWT sperm exposed to ASP (A,B) and a hypotonic solution (C,D). (A–D) Autoradiographs of ^{32}P-labelled proteins. (E,F) Coomassie Brilliant Blue staining of sperm proteins in ASP. IEF, isoelectric focusing.

assays were conducted using ^{32}P (as $H_3{}^{32}PO_4$) to investigate the phosphorylation reactions that occur in response to osmotic stress. We detected phosphorylation reactions that occurred when sperm were transferred to hypotonic or hypertonic solutions from isotonic artificial seminal plasma (ASP). In FWT sperm, the ^{32}P-labelled protein signal decreased when sperm were suspended in a hypotonic solution (50 mmol l^{-1} NaCl, 5 mmol l^{-1} $CaCl_2$, 10 mmol l^{-1} HEPES–NaOH, pH 8.0) that activates motility, suggesting that protein dephosphorylation occurred (Fig. 1A). Protein dephosphorylation did not occur in a hypertonic solution (300 mmol l^{-1} NaCl, 10 mmol l^{-1} $CaCl_2$, 10 mmol l^{-1} HEPES–NaOH, pH 8.0; Fig. 1A) that reduces motility. In contrast, in SWT sperm, dephosphorylation of sperm proteins was observed in both hypotonic and hypertonic solutions, in which SWT sperm were motile (Fig. 1A). The incorporation of ^{32}P into 40-kDa proteins in SWT sperm during incubation in ASP was slightly stronger than in FWT sperm (Fig. 1A; $P<0.05$ *t*-test).

FWT and SWT sperm are specialised to tolerate different osmotic environments so that they will respond to osmotic stimuli that activate flagellar motility. To determine the involvement of protein phosphorylation in the activation of motility more precisely, sperm proteins were separated into a Triton-soluble fraction, which contains some membrane proteins and the cytoplasm, and remnants (i.e. the remaining flagellar proteins after extraction with detergent and high salt solutions) to determine their roles in the activation of motility. In the Triton-soluble fraction, 18 kDa proteins of FWT sperm were strongly phosphorylated (Fig. 1A,C). These 18 kDa proteins were dephosphorylated following exposure of the sperm to a hypotonic solution (Fig. 1A–C). In SWT sperm, the ratio of densitogram [(18 kDa in hypotonic or hypertonic solutions/18 kDa

in ASP)] indicates that desphosphorylation of the 18 kDa protein also occurred in hypertonic solution (Fig. 1D). The strength of the phosphorylated state of the 18 kDa band was different between FWT and SWT sperm (Fig. 1A–C), thus we calculated the ratio of densitogram to detect changes in the band intensity when sperm were transferred to the hypotonic or hypertonic solutions.

Next, ^{32}P-labelled proteins were separated by 2-D electrophoresis. The 18 kDa proteins were resolved into three spots. The 18 kDa proteins were more phosphorylated in FWT sperm than in SWT sperm (Fig. 1C, Fig. 2A,B). In SWT sperm, 40 to 65 kDa proteins were very phosphorylated (Fig. 2B). ^{32}P-labelled proteins were dephosphorylated after sperm were transferred to a hypotonic solution (Fig. 1D, Fig. 2C,D). Notably, patterns of phosphorylation of the 18 kDa proteins in the Triton-soluble fraction were different (Fig. 1B). Presumably, osmotic shock causes activation of phosphorylation cascades at the plasma membrane. In addition, some proteins that are involved in the reactions may be present in the Triton-soluble fraction. Thus, the difference in the phosphorylation reactions of the 18 kDa proteins in the Triton-soluble fraction between SWT and FWT sperm could reflect adaptation to different osmotic environments.

Identification of the 18-kDa proteins

Osmotic shock that induces motility activation in sperm may be different depending on the hypotonic and hypertonic aquatic environment. In support of this proposal, we found that the phosphorylation patterns of the 18 kDa proteins were different in FWT and SWT sperm (Fig. 1A,B, Fig. 2A–D). The amino acid sequence of the phosphorylated 18 kDa protein contained the sequence VLKAVTVLKGTGDTSGTVYF. This 20-residue sequence has 95 and 85% homology to the GenBank-listed N-terminal region of Cu/Zn superoxide dismutase (Cu/Zn SOD) from tilapia *O. mossambicus* and the grouper *Epinephelus malabaricus*, respectively. The sixth residue (threonine) corresponds to a cysteine residue in the *O. mossambicus* cDNA (GenBank, AY491056). A polyclonal anti-peptide antibody was raised against the N-terminal region of this 18 kDa protein. This antibody recognised the 18 kDa proteins, which separated into two spots (Fig. 3A). The ^{32}P-labelled 18 kDa proteins were separated into three spots and isoelectric points of two spots were similar to those of 18 kDa antibody-positive spots (Fig. 2A,B, Fig. 3A). One basic spot did not coincide with the 18 kDa antibody-positive spots. Therefore, the basic 18 kDa spot is likely to be distinctive protein. The 18-kDa protein was present in the Triton-soluble fractions of the flagellum and the sleeve structure (Fig. 3B). However, immunofluorescence analysis with anti-18 kDa and Cu/Zn SOD antibodies failed to stain SOD in the sperm. Immunoprecipitation was performed using the anti-18 kDa and anti-Cu/Zn SOD antibodies to confirm whether the 18-kDa protein was Cu/Zn SOD. The anti-Cu/Zn SOD antibody reacted with a precipitate obtained using the 18-kDa antibody (Fig. 3C), suggesting that the 18 kDa protein was indeed Cu/Zn SOD. Furthermore, a ^{32}P-labelled 18-kDa protein was detected in immunoprecipitates obtained using the anti-18 kDa antibody (Fig. 4), which suggests that the phosphorylated 18 kDa protein was Cu/Zn SOD. Thus, the FWT-specific 18 kDa phosphoprotein might be Cu/Zn SOD. Its pattern of phosphorylation was modulated during acclimatisation of the fish to different osmotic environments.

To confirm that Cu/Zn SOD was present in the sperm cells, SOD activity was measured in the sleeves and flagella. SOD activity was detected in both the sleeve and the flagellum fractions (Fig. 5). Cross-linking experiment showed that Cu/Zn SOD interacted with other proteins (Fig. 6). Together, these data suggest that Cu/Zn SOD potentially remains active in the flagellum and interacts with other proteins.

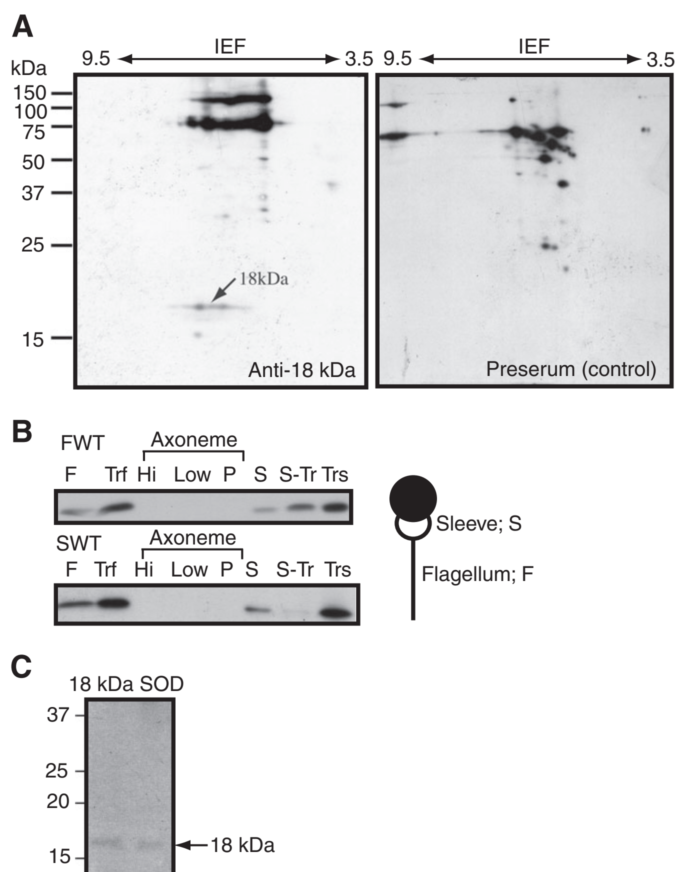

Fig. 3. Identification of the 18 kDa proteins as Cu/Zn superoxide dismutase (SOD). (A) The 18-kDa protein was detected by the anti-18-kDa peptide antibody after sperm proteins were separated by 12.5% 2-D SDS–PAGE. (B) The 18-kDa protein was detected after sperm proteins were fractionated. Lanes: F, the flagellar fraction; Hi, high ion (0.6 mol l^{-1} NaCl) extract; Low, low ion extract; P, the remnant of the axoneme; S, sleeve proteins separated from the Triton-soluble fraction; S-Trs, the Triton-soluble fraction minus the sleeve proteins; Trf, Triton-soluble fraction containing the flagella proteins; TRS, Triton-soluble fraction. (Right) A diagram of a sperm to show the position of the sleeve. (C) Immunoprecipitation was conducted using anti-18-kDa and anti-Cu/Zn SOD antibodies. The anti-Cu/Zn SOD antibody reacted with a precipitate obtained using the 18-kDa antibody.

Reactive oxygen species production in sperm

Reactive oxygen species (ROS) function as second messengers that modulate sperm protein function (de Lamirande et al., 1997; Aitken et al., 1998; Herrero and Gagnon, 2001; O'Flaherty et al., 2006). In mammalian sperm, peroxynitrite ($ONOO^-$), which is formed by the reaction of nitric oxide (NO) with the superoxide anion (O_2^-), attaches to tyrosine residues in sperm proteins ('nitration'), thereby affecting capacitation (Aitken et al., 1995; de Lamirande and Gagnon, 1995; de Lamirande et al., 1998a; de Lamirande et al., 1998b; Belen Herrero et al., 2000; de Lamirande and Gagnon, 2002). The amount of O_2^- present determines the amount of $ONOO^-$ produced (Wink and Mitchell, 1998). Thus, SOD is potentially involved in the production of $ONOO^-$. Moreover, SOD potentially catalyses tyrosine nitration (Beckman et al., 1992; Ischiropoulos et al., 1992; Lymar et al., 1996; Crow et al., 1997). To examine the role of $ONOO^-$ in the activation of motility in FWT and SWT sperm

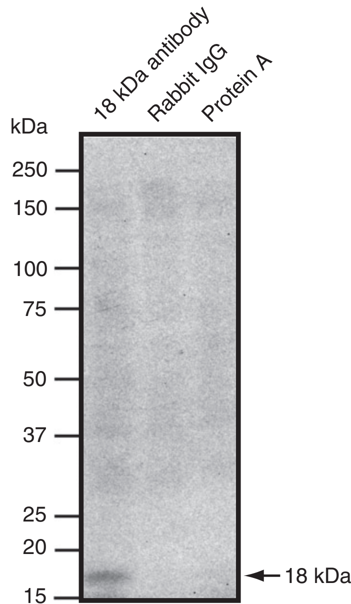

Fig. 4. Identification of the phosphorylated 18 kDa protein as a Cu/Zn SOD. Immunoprecipitation was conducted using the anti-18 kDa antibody, anti-rabbit IgG and protein-A–Sepharose against ^{32}P-labelled proteins (Triton-soluble fraction). Precipitates were separated by 12.5% SDS–PAGE and the ^{32}P-labelled proteins were detected.

in response to osmotic shock, the nitration of tyrosine residues in sperm proteins as a result of osmotic shock was investigated.

The nitration of tyrosine residues in sperm flagellar proteins, Triton-soluble fractions and high salt-soluble fractions were altered by osmotic shock, and the nitration reactions were found to be different in FWT and SWT sperm (Fig. 7A–F). In FWT sperm flagellar proteins, nitration occurred under hypotonic conditions that activate motility (Fig. 7A). In contrast, a decrease in nitration signals was observed in SWT sperm flagellar proteins under hypotonic and hypertonic conditions that reduce motility (Fig. 7D). To examine the localization of nitrated proteins, sperm flagellar proteins were successively eluted with a Triton X-100 solution and a high salt solution (0.6 mol l^{-1} NaCl) after sperm were exposed to hypotonic solution or ASP. Nitration in the Triton-soluble fractions of FWT and SWT sperm flagella was different (Fig. 7B,E), whereas that in the high salt fractions was similar (Fig. 7C,F). Therefore, nitration reactions in the axonemes are likely to be identical in FWT and SWT sperm. High-salt extraction causes the outer dynein arms to be solubilised. Thus, it was to be expected that outer dynein arms

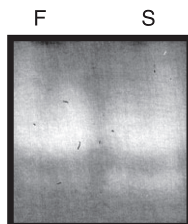

Fig. 5. SOD activity in sperm. SOD activity in sperm that were in isotonic ASP was detected according to the method of Flohe and Otting (Flohe and Otting, 1984). SOD activity was measured in the Triton-soluble fraction of the sleeve structure (S) and flagellum (F) after proteins were separated by native PAGE (7.5% gel).

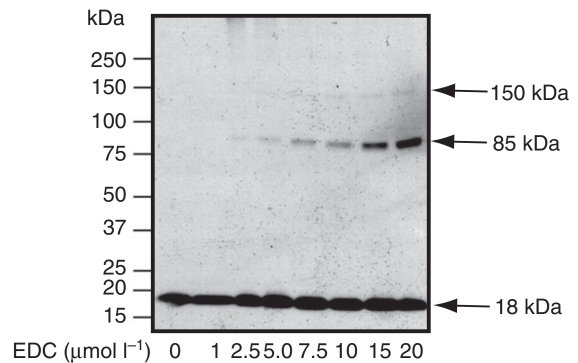

Fig. 6. Detection of proteins associated with SOD. To detect proteins associated with SOD, cross-linking was conducted using a range of concentrations of 1-ethyl-3-[3-dimethylaminopropyl]carbodiimide hydrochloride (EDC). Cross-linked Triton-soluble proteins from FWT sperm were separated by 12.5% SDS–PAGE, and immunoblotting was performed with the anti-Cu/Zn SOD antibody.

from tilapia sperm flagellar axonemes would be present in the high salt fractions. The outer dynein arms in *Chlamydomonas* spp. are regulated in a redox-dependent manner (Wakabayashi and King, 2006), whereas the outer dynein complex in the flagella of sperm from the prochordate *Ciona intestinalis* contains proteins that have a ROS-sensitive thioredoxin domain (Hozumi et al., 2006). It is possible that the outer dynein arm in the tilapia axoneme is also regulated in a redox-dependent manner. However, further study is required to show any relevant ROS-dependent regulation of outer dynein arms.

DISCUSSION

Our data demonstrate that ROS-dependent signal transduction correlated with the activation of flagellar motility in tilapia sperm. Differences in nitration and SOD phosphorylation between FWT and SWT sperm proteins are closely linked to osmotic adaptations in sperm cells (Figs 1, 2, 7). Our study demonstrates that SOD closely interacts with proteins in tilapia sperm (Fig. 6). Mutant form of Cu/Zn SOD has previously been reported to interact with heat-shock protein (HSP) 70 and HSP40, and αB-crystallin (Shinder et al., 2001). Several studies have shown that flagella and cilia contain HSP70 (Bloch and Johnson, 1995; Stephens, 1997; Williams and Nelsen, 1997; Stephens and Lemieux, 1999), HSP90 (Williams and Nelsen, 1997; Stephens and Lemieux, 1999) and HSP40 (Satouh et al., 2005; Yang et al., 2005; Yang et al., 2008). Furthermore, flagellar motility is affected by the redox-dependent regulation of the outer dynein arms (Wakabayashi and King, 2006). It is possible that SOD interacts with HSPs in the flagellum and that redox-dependent regulation contributes to flagellar motility. However, SOD was not present in the axoneme (Fig. 3B), although it could be interacting with other proteins (Fig. 6), thus, further investigation is required to more precisely identify roles of SOD in the regulation of flagellar motility in tilapia sperm.

Redox-dependent regulation of motility is well established in mammalian sperm. Although, in large quantities, ROS such as NO, O_2^- and hydrogen peroxide are toxic (Storey, 1997; Aitken et al., 1998), in mammalian sperm, small amounts of ROS play a beneficial role in capacitation and the regulation of motility (Aitken et al., 1995; de Lamirande and Gagnon, 1995; de Lamirande et al., 1997; Aitken et al., 1998; de Lamirande and Gagnon, 1998; de Lamirande et al., 1998a; Herrero et al., 2000; Herrero and Gagnon, 2001;

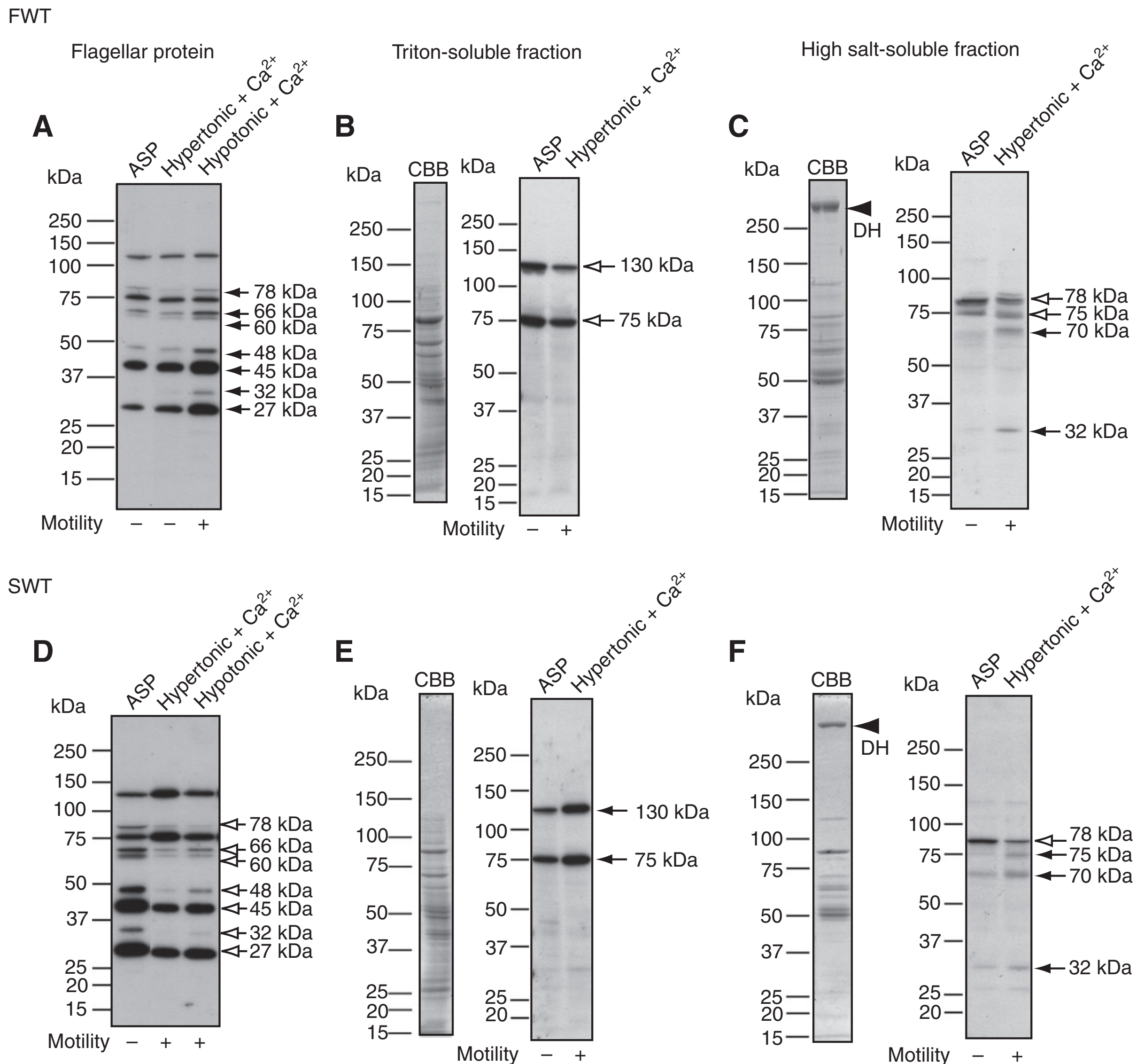

Fig. 7. Protein nitration of tyrosine residue(s) in sperm flagella. FWT and SWT sperm were suspended in ASP, in a hypertonic solution plus Ca^{2+} (300 mmol l^{-1} NaCl + 5 mmol l^{-1} $CaCl_2$, or in a hypotonic solution plus Ca^{2+} (50 mmol l^{-1} NaCl + 5 mmol l^{-1} $CaCl_2$). Flagella and sleeves were separated and isolated flagella from FWT and SWT sperm were subjected to western analyses to detect nitrotyrosine with polyclonal anti-nitrotyrosine antibody (A,D). To separate flagellar proteins into a Triton-soluble fraction and a high-salt-soluble fraction, isolated flagella from sperm, which were suspended in ASP or hypotonic solution, were demembranated with NP-40 to obtain axonemes. The proteins extracted by demembranation are shown in B,E (Triton-soluble fraction). The axonemes were successively treated with 0.6 mol l^{-1} NaCl (C,F: high-salt-soluble fraction). Outer dynein arms should be present in the high-salt-soluble fraction. The dynein heavy chain band is indicated by a filled arrowhead in the Coomassie Brilliant Blue (CBB) gel. Flagellar proteins and fractions were separated by SDS-PAGE using a 5–15% gradient polyacrylamide gel. Filled arrows indicate that the tyrosine residues of these proteins were nitrated when sperm were exposed to hypotonic or hypertonic solutions. Open arrows indicate a decrease in nitrated signals when sperm were exposed to hypotonic or hypertonic solutions.

O'Flaherty et al., 2006). ROS production have been reported in the sperm of mammals. ROS are primarily produced as a result of mitochondrial respiration, which supplies the ATP necessary for the maintenance of motility. A by-product of ATP production in mitochondria, O_2^-, is generated as the result of NADPH dehydrogenase activity (Turrens and Boveris, 1980). Flagellar motility is sustained by ATP derived from the mitochondria, indicating that O_2^- is generated in parallel with flagellar motility. In contrast, other animals use ATP derived from phospholipids and glycogen rather than from mitochondria (Mita and Yasumasu, 1989; Mukai and Okuno, 2004). In sea urchin sperm, a creatine-kinase-dependent ATP supplementation mechanism operates along the flagellum (Tombes and Shapiro, 1985). Moreover, O_2^- that is generated during respiration is quickly scavenged by manganese SOD (MnSOD) in the mitochondria. As a result, only very small amounts of O_2^- leak out of the mitochondria. However, the leakage of O_2^- from mitochondria has been reported (Aitken, 1997; Vernet et al., 2001). Additionally, membrane-bound NADPH has been detected in mammalian sperm (Aitken, 1997; Aitken et al., 2003). There are several probable sources of O_2^- in sperm cells but these have not been precisely determined. O_2^- generated in sperm is scavenged by SOD, which is constitutively active in sperm cells (Figs 4, 5). The implications of the apparent differences in SOD phosphorylation state between FWT and SWT sperm (Figs 1, 2)

remain unclear. However, they appear likely to be associated with osmotic tolerance and the regulation of motility. Moreover, redox-dependent signals may contribute to differences in the osmotic resistance of sperm cells.

It is possible that ROS, including O_2^-, play an important role in the regulation of motility. $ONOO^-$, which drives the nitration of tyrosine residues in proteins, is a candidate activator of motility (Greenacre and Ischiropoulos, 2001; Turko and Murad, 2002). Although SOD restricts $ONOO^-$ formation through the dismutation of O_2^-, it catalyses nitration (Schopfer et al., 2003). Nitration affects protein status and contributes to several signal transduction pathways in mammalian sperm (Aitken et al., 1995; de Lamirande and Gagnon, 1995; de Lamirande et al., 1997; Aitken et al., 1998; de Lamirande and Gagnon, 1998; de Lamirande et al., 1998a; Herrero et al., 2000; Herrero and Gagnon, 2001; O'Flaherty et al., 2006). In mammalian sperm, nitration affects tyrosine phosphorylation, which is involved in capacitation (Aitken et al., 1995; de Lamirande et al., 1997; O'Flaherty et al., 2006). Our results demonstrate that the nitration and denitration of tilapia sperm flagellar proteins coincide with motility activation (Fig. 7A,D). Moreover, differences in phosphorylation between FWT and SWT sperm are potentially associated with differences in nitration. As well as being linked to nitration (Beckman et al., 1992; Ischiropoulos et al., 1992; Lymar et al., 1996; Crow et al., 1997), SOD interacts latently with HSPs (Shinder et al., 2001), which consist of a radial spoke and central pair system that may regulate dynein activity (Bloch and Johnson, 1995; Stephens, 1997; Williams and Nelsen, 1997; Satouh et al., 2005; Yang et al., 2005; Yang et al., 2008). Thus, SOD may play an important role in the regulation of sperm motility by reducing $ONOO^-$ formation or nitration. It is possible that SOD mediates nitration events, and that differences in nitration lead to changes in sperm motility when fish move from freshwater to seawater.

Hydrogen peroxide, produced from O_2^- *via* SOD activity, is itself toxic. Thus, catalase or peroxiredoxin, which convert hydrogen peroxide to water (Chelikania et al., 2004; Rhee et al., 2005), should be expressed alongside SOD. Catalase and peroxiredoxin are present in sperm cells (van Gestel et al., 2005; Mansour et al., 2006) and seminal fluid (Lapointe et al., 1998). Water may be transported by aquaporins in the plasma membrane (King and Agre, 1996; Verkman, 2002). Sperm of the marine teleost sea bream (*Sparus aurata*) express aquaporin 1a and aquaglyceroporin, which are localised to the plasma membrane of the head and flagellum (Zilli et al., 2009). In tilapia sperm, it seems reasonable that redox-dependent signal cascades are involved in the regulation of osmotic tolerance and motility. However, further analyses at the molecular level are required to determine the precise mechanisms.

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