Mitochondrial aquaporin-8-mediated hydrogen peroxide transport is essential for teleost spermatozoon motility

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Reactive oxygen species (ROS), particularly hydrogen peroxide (H₂O₂), cause oxidative cell damage and inhibit sperm function. In most oviparous fishes that spawn in seawater (SW), spermatozoa may be exposed to harmful ROS loads associated with the hyperosmotic stress of axonemal activation and ATP synthesis from mitochondrial oxidative phosphorylation. However, it is not known how marine spermatozoa can cope with the increased ROS levels to maintain flagellar motility. Here, we show that a marine teleost orthologue of human aquaporin-8, termed Aqp8b, is rapidly phosphorylated and inserted into the inner mitochondrial membrane of SW-activated spermatozoa, where it facilitates H₂O₂ efflux from this compartment. When Aqp8b intracellular trafficking and mitochondrial channel activity are immunologically blocked in activated spermatozoa, ROS levels accumulate in the mitochondria leading to mitochondrial membrane depolarisation, the reduction of ATP production, and the progressive arrest of sperm motility. However, the decreased sperm vitality underlying Aqp8b loss of function is fully reversed in the presence of a mitochondria-targeted antioxidant. These findings reveal a previously unknown detoxification mechanism in spermatozoa under hypertonic conditions, whereby mitochondrial Aqp8b-mediated H₂O₂ efflux permits fuel production and the maintenance of flagellar motility.

Spermatozoon motility is a major physiological determinant of male fertility. Amongst external fertilizers, such as freshwater and marine fishes, activation of motility is respectively induced by the hypo- or hyper-osmotic aquatic environment into which the sperm are ejaculated1–4. Spermatozoon flagellar motility is primarily driven by the hydrolysis of ATP, which is synthesised from glycolysis and/or mitochondrial oxidative phosphorylation (OXPHOS) depending on the species5–9. Due to electron leakage from the mitochondrial electron transport chain during OXPHOS10, however, or directly resulting from the osmotic stress of activation11–15, reactive oxygen species (ROS), such as hydrogen peroxide (H₂O₂), may be produced in excess causing depolarisation of the mitochondrial membrane potential (ΔΨₘ) and mitochondrial malfunctioning10,16,17. As a result, spermatozoa enter into oxidative stress, which may lead to membrane lipid peroxidation, depletion of ATP, or axoneme damage, thus inhibiting sperm motility18–22.

In marine teleosts, oxidative damage in spermatozoa linked to osmotic stress may be particularly critical since these cells face a strong hyperosmotic shock (from ~300 to ~1100 mOsm) when they are released into seawater (SW). Such osmotically-induced H₂O₂ may subsequently diffuse into the mitochondrion further exacerbating the toxic effects of ROS. As in mammals, however, the normal detoxification pathways involving antioxidants, enzymes and peroxisomes that are present in somatic cells23, are more limited in spermatozoa24–26, due in part to the relatively low cytoplasmic volume following spermiogenesis and the transcriptional quiescence of the germ cells27. Consequently, it is not known how marine fish spermatozoa maintain motility and thus a fertilisation potential during high endogenous production of ROS.

In recent years, aquaporin homologues from plants and animals that facilitate transmembrane water transport, have also been identified as H₂O₂ channels28–33. Amongst these homologues is human aquaporin-8 (AQP8), which also transports ammonia34, and has been shown to be present in the inner mitochondrial membrane of hepatic35 and renal proximal tubule cells36, where it is suggested to mediate ammonia and H₂O₂ transport37,38 rather than water fluxes39,40. However, direct evidence that mitochondrial AQP8 mitigates cellular oxidative stress in a physiological framework has not yet been reported.
In the oviparous marine teleost, gilthead seabream (*Sparus aurata*), a recent study revealed that upon SW activation of spermatozoa, the teleost orthologue of AQP8, termed Aqp8b, is rapidly accumulated in the single mitochondrion of these cells. In the present work, we therefore selected the seabream as a model to test the hypothesis that H$_2$O$_2$ transport through mitochondrial Aqp8b may be essential for the maintenance of motility of marine spermatozoa. Our data show that seabream Aqp8b operates as a mitochondrial peroxiporin in activated sperm and that immunological inhibition of channel function causes mitochondrial failure and the decline of motility, whereas these effects are fully reversed using a mitochondria-targeted antioxidant. These findings uncover a novel aquaporin-mediated detoxification mechanism in spermatozoa and demonstrate a physiological role of mitochondrial AQP8-facilitated H$_2$O$_2$ transport.

**Results**

**Seabream Aqp8b is phosphorylated and transported to the spermatozoon mitochondrion upon activation.** The mature spermatozoon of seabream is a uniflagellated cell, differentiated into an acrosome-less head, a short midpiece and a long cylindrical flagellum bearing the microtubule-forming axoneme (Fig. 1a). In the midpiece region, a single large mitochondrion is present, which is typical of the spermatozoon of many members of the family Sparidae. Both the flagellum and mitochondrion can be respectively labelled with anti-α-tubulin antibodies and the mitochondria-specific vital dye MitoTracker (Fig. 1b). Immunofluorescence microscopy of ejaculated spermatozoa maintained in non-activating medium (NAM), using the MitoTracker and an affinity-purified antibody against the seabream Aqp8b C-terminus, indicated that Aqp8b is located in the midpiece region and anterior part of the flagellum in immotile sperm, whereas the channel accumulates in the mitochondrion upon SW activation (Fig. 1c). Immunoblotting of whole sperm in NAM detected a single reactive band with the expected size of the Aqp8b monomer (28 kDa), while an additional band of slightly higher molecular mass (Fig. 1d) was detected within 1 s of exposure of spermatozoa to SW (Fig. 1d). Subcellular fractionation of spermatozoa and further immunoblotting of Aqp8b together with markers for the inner and outer mitochondrial membrane and mitochondrial matrix, prohibitin (Phb), hexokinase 1 (Hk1) and heat shock protein 60 (Hsp60), respectively, revealed a higher prevalence of both Aqp8b immunoreactive bands in the mitochondrion of activated sperm, thus confirming the immunostaining data, while only a single higher molecular mass band was detected in the inner mitochondrial membrane subcompartment (Fig. 1e). Further treatment of the whole mitochondrial extract from SW spermatozoa with alkaline phosphatase reduced the intensity of the 30-kDa Aqp8b reactive band (Fig. 1f), suggesting that this band most likely corresponds to a phosphorylated form of Aqp8b. These data indicate that Aqp8b is phosphorylated and rapidly trafficked (<1 s) to the inner mitochondrial membrane of the seabream spermatozoon upon SW activation.

Figure 1: Aqp8b is phosphorylated and rapidly accumulated in the mitochondrion of the seabream spermatozoon upon SW activation. (a) Schematic diagram of the seabream spermatozoon. (b, c) Immunolocalisation of α-tubulin (Tuba) in immotile spermatozoon (green in b), and Aqp8b (green in c) in non-activated and SW-activated spermatozoa. In (b) and (c), the spermatozoon nucleus and mitochondrion were counterstained with DAPI (blue) and the mitochondrial dye MitoTracker (red), respectively. Scale bars, 1 μm. (d) Representative Western blot of Aqp8b, and Tuba as loading control, in non-activated and activated spermatozoa for 1, 5 or 10 s. (e) Aqp8b immunoblot of mitochondria and mitochondrial inner membrane isolated from immotile and motile spermatozoa. Phb and Hk1 were used as markers of inner and outer mitochondrial membrane, respectively, and Hsp60 as marker of the mitochondrial matrix. (f) Dephosphorylation of mitochondrial Aqp8b by phosphatase treatment. Phosphorylated (p-Aqp8b) and dephosphorylated Aqp8b are indicated. Hk1 was used as loading control. Full-length blots in (d–f) are presented in Supplementary Fig. S3 online. H, head; F, flagellum; Mp, midpiece; N, nucleus; Mc, mitochondria; IMM, inner mitochondrial membrane; CIP, calf intestine alkaline phosphatase; MTR, MitoTracker.
Seabream Aqp8b facilitates H₂O₂ uptake in oocytes and sperm mitochondria.

Previous studies have shown that the seabream Aqp8b is permeable to water and urea, but not to glycerol⁴⁰. Amino acid alignment of seabream Aqp8b with human AQP8, which has been shown to transport H₂O₂,²⁶,³², revealed that three of the four Aqp8b residues (H72, C208 and R213) in the aromatic arginine region (ar/R) at the extracellular pore mouth that have been suggested to allow the passage of H₂O₂⁴⁰, are conserved⁴⁰. To experimentally test whether the seabream Aqp8b can conduct H₂O₂, we used the Xenopus laevis oocyte expression system, which yields good expression of the channel at the oocyte plasma membrane (Fig. 2a, b). We first evaluated volumetrically whether the Aqp8b-Ab was able to block Aqp8b-mediated water transport. The osmotic water permeability (Pₜ) of Aqp8b-expressing oocytes increased by seven-fold with respect to water-injected (control) oocytes, while mercury reduced the Pₜ by 93 ± 4% (Fig. 2c; Supplementary Fig. S1 online). Interestingly, externally added Aqp8b-Ab in the presence of 0.5% Me₂SO (DMSO) inhibited oocyte water permeability in a dose-dependent manner up to 67 ± 10%, whereas rabbit IgG was ineffective (Fig. 2c; Fig. S1 online). The effect of the Aqp8b-Ab was specific since the Pₜ of oocytes expressing Aqp1aa, -1ab, -7 or -10b cRNAs (1–5 ng) was not affected by the Aqp8b-Ab treatment, whereas the Pₜ was inhibited (59–74%) when oocytes were exposed to the corresponding antibodies (Fig. 2d).

Seabream Aqp8b facilitates H₂O₂ uptake in oocytes and sperm mitochondria.

The uptake of H₂O₂ by control and seabream Aqp8b-expressing oocytes was subsequently determined using the ROS-sensitive, cell-permeable fluorescent dye 5-(and-6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate, acetyl ester (CM-H₂DCFDA), which has previously been employed to evaluate H₂O₂ transport in yeast cells transformed with heterologous aquaporins as well as in AQP3-expressing mice T cells. For these experiments, control and Aqp8b oocytes were loaded with CM-H₂DCFDA and the increase in oocyte fluorescence following exposure to increasing concentrations of external H₂O₂ was determined spectrophotometrically. Exogenous H₂O₂ supplementation for 30 min significantly increased fluorescence intensity in a dose-response manner in Aqp8b oocytes, the increase being approximately three-fold higher with respect to controls upon addition of 100 μM H₂O₂ (Fig. 3a). The increase in fluorescence of Aqp8b oocytes was significantly reduced by 72 ± 5% and 90 ± 4% by bodies revealed specific signals in the plasma membrane and the cytoplasm, indicating that exogenously added antibody was able to bind Aqp8b in both compartments (Fig. 2e, right panel). The binding of the antibody was confirmed by protein G coprecipitation experiments, which detected Aqp8b-Ab-Aqp8b complexes only in oocytes expressing Aqp8b and treated with Aqp8b-Ab (Fig. 2f). Immunoblotting of total and plasma membrane fractions of these oocytes also showed lower amounts of Aqp8b in the plasma membrane when compared with those exposed to IgG (Fig. 2g), suggesting that the Aqp8b-Ab partially prevented the insertion of the channel into the oocyte surface.

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Aqp8b-Ab and HgCl$_2$, respectively, while IgG had no effect (Fig. 3a), suggesting the involvement of Aqp8b in H$_2$O$_2$ transport in oocytes. To corroborate that Aqp8b inserted in the mitochondrion of the seabream spermatozoa was able to facilitate H$_2$O$_2$ transport, the uptake of exogenous peroxide by isolated and CM-H$_2$DCFDA-loaded mitochondria from SW-activated sperm or from immotile sperm maintained in NAM, was estimated as for oocytes. Exogenous peroxide addition resulted in a significant increase of fluorescence intensity of both SW and NAM mitochondria in a dose-response manner, but the increase in fluorescence was much higher in mitochondria from SW-activated sperm (489 $\pm$ 18%) compared to mitochondria from NAM spermatozoa (131 $\pm$ 2%) (Fig. 3b). Also as observed in oocytes, the fluorescence intensity of SW-activated mitochondria stimulated with 300 $\mu$M H$_2$O$_2$ was respectively blocked by 78 $\pm$ 1% and 84 $\pm$ 2% by the Aqp8b-Ab and mercury, and unaffected by the DMSO vehicle or IgG treatment (Fig. 3c). In contrast, the peroxide-induced slight increase in fluorescence of mitochondria from immotile sperm remained unchanged after addition of either of the Aqp8b inhibitors (Fig. 3c). Protein-G precipitation from SW mitochondrial extracts treated with the Aqp8b-Ab pulled down the phosphorylated and dephosphorylated Aqp8b bands, demonstrating the binding of the antibody with endogenous Aqp8b (Fig. 3d). These findings demonstrate that mitochondrial Aqp8b in activated seabream spermatozoa facilitates H$_2$O$_2$ transport. In addition, these and previous data indicate that the Aqp8b-Ab can block the trafficking of Aqp8b when heterologously expressed in oocytes, as well as the permeability of the endogenous channel inserted in the spermatozoan inner mitochondrial membrane.

**Mitochondrial Aqp8b is required for spermatozoa motility.** It has been established that spermatozoa have a limited amount of RNA and are transcriptionally silent$^{27}$, although long-lasting mRNA molecules transcribed during spermatogenesis can be translated in spermatozoa by mitochondrial ribosomes$^{42}$. However, in the ejaculated sperm of seabream, aqp8b transcripts have not been detected$^{40}$. Therefore, we employed the Aqp8b-Ab to investigate the functional role of Aqp8b during sperm motility in SW. Initial trials indicated that the same concentrations of exogenous antibody...
tested on oocytes specifically reduced both the percentage of motile and progressive spermatozoa during the 5–10 s interval post-activation in a dose-response manner (Fig. S2). Subsequent time-course experiments up to 3 min revealed that the Aqp8b-Ab drastically reduced the temporal motility (Fig. 4a) and progression (Fig. 4b) of activated spermatozoa with respect DMSO- and IgG-treated sperm (controls). In addition, the Aqp8b-Ab affected other kinetic parameters related to the vigour of spermatozoa, such as curvilinear velocity and beat cross frequency (Fig. 4c, d), and progressiveness, such as the straight-line velocity and linearity (Fig. 4e, f).

The staining of spermatozoa treated with IgG or Aqp8b-Ab with MitoTracker and either FITC-labelled anti-IgG or Alexa fluor 488-conjugated Aqp8b-Ab (0.3 μM), which were immunostained (green) with only fluorescein isothiocyanate-labelled anti-IgG or Alexa fluor 488-conjugated DAPI nuclear stain (blue), were significantly different (P indicated in each panel). (g) Merged images of IgG- and Aqp8b-Ab-treated spermatozoa, labelled with MitoTracker (red) and DAPI nuclear stain (blue), which were immunostained (green) with only fluorescein isothiocyanate-labelled anti-IgG or Alexa fluor 488-conjugated Aqp8b-Ab. Scale bars, 2 μm. (h) Protein G coprecipitation of Aqp8b-Ab-Aqp8b complexes in whole sperm and mitochondria from activated spermatozoa treated with 0.3 μM IgG or Aqp8b-Ab. (i) Immunoblot of mitochondrial Aqp8b from SW-activated sperm treated as in (h) using Hsp60 as indicated in each panel). (j) Quantitation (mean ± SEM; n = 5 fish) of mitochondrial Aqp8b in (i) normalised to Hsp60, which did not change between treatments (P = 0.291).

These findings suggested that Aqp8b function in the spermatozoon mitochondrion is necessary to prevent ROS accumulation and depolarisation of the ΔΨm in order to facilitate ATP production.

**Aqp8b functions as a mitochondrial peroxiporin for H2O2 efflux during OXPHOS in vitro.** To investigate further whether Aqp8b can mediate mitochondrial ROS efflux, we measured the production of ATP, the concomitant ROS generation and the variations in the ΔΨm during OXPHOS in vitro by mitochondria isolated from activated sperm. A robust production of ATP was observed after addition of exogenous ADP, that was completely abolished by the ATP synthase inhibitor oligomycin, which confirmed the functionality of the ATP assay (Fig. 6a). The addition of the Aqp8b-Ab however reduced the production of ATP by 38 ± 3%, whereas IgG had no effect (Fig. 6a).

The generation of ROS estimated with the CM-H_2DCFDA dye during ATP production in vitro, indicated that DMSO- and IgG-treated mitochondria enhanced both ROS accumulation (by 176 ± 6% and 107 ± 6%, in DMSO and IgG sperm, respectively) (Fig. 5c). However, in Aqp8b-Ab-treated sperm the increase in fluorescence was significantly higher (215 ± 13%) (Fig. 5c), suggesting a potential oxidative stress scenario in spermatozoa linked to the inhibition of mitochondrial Aqp8b. Interestingly, evaluation of the ΔΨm with the lipophilic cationic compound 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolyl carbocyanine iodine (JC-1), which was specific for the mitochondrial ROS efflux, we measured the production of ATP, the concomitant ROS generation and the variations in the ΔΨm during OXPHOS in vitro by mitochondria isolated from activated sperm. A robust production of ATP was observed after addition of exogenous ADP, that was completely abolished by the ATP synthase inhibitor oligomycin, which confirmed the functionality of the ATP assay (Fig. 6a). The addition of the Aqp8b-Ab however reduced the production of ATP by 38 ± 3%, whereas IgG had no effect (Fig. 6a).
15% and 173 ± 20%, respectively) and release (by 220 ± 19% and 221 ± 25%, respectively) (Fig. 6b). In contrast, ROS efflux appeared to be impaired in mitochondria exposed to the Aqp8b-Ab with respect to the controls (147 ± 15%), leading to a higher increase in ROS accumulation (223 ± 10%) (Fig. 6b). Such mitochondrial ROS accumulation was associated with a decrease of the $\Delta \Psi_m$ (by 22 ± 2%) compared to the control groups (Fig. 6c). Addition of the mitochondria-targeted antioxidant mito-TEMPO, which has superoxide and alkyl scavenging properties, reduced the generation of ROS in all treatment groups (Fig. 6b), but interestingly had no effect on the $\Delta \Psi_m$ (Fig. 6c), the ATP content (Fig. 6d) and the ATP production ATP (Fig. 6d) to control levels. However, in mitochondria treated with the Aqp8b-Ab the antioxidant restored both the $\Delta \Psi_m$ (Fig. 6c) and the production of ATP (Fig. 6d) to control levels.

The antioxidant mito-TEMPO rescues ATP production and motility of Aqp8b-Ab-treated spermatozoa. Treatment with mito-TEMPO completely reversed the Aqp8b-Ab inhibition on mitochondrial ATP production in vitro, and we therefore tested further if this antioxidant could prevent the negative actions of Aqp8b-Ab on sperm functions during activation. Consistent with previous results on isolated mitochondria, addition of the antioxidant to IgG-treated spermatozoa was able to reduce the ROS levels (Fig. 7a), but had no effect on the $\Delta \Psi_m$ (Fig. 7b), the ATP content (Fig. 7c), or the different parameters related to spermatozoa motility (Fig. 7d–i). On the contrary, mito-TEMPO-mediated reduction of ROS in Aqp8b-Ab-treated sperm reversed the decrease of the $\Delta \Psi_m$ and ATP content induced by the antibody (Fig. 7a–c), and fully rescued all spermatozoa motility parameters (Fig. 7d–i). These data therefore suggest that the harmful effect on flagellar motility resulting from the inhibition of the Aqp8b function is caused by the accumulation of ROS in the spermatozoa mitochondrion.

**Discussion**

In this study, we show that mitochondrial Aqp8b plays a vital detoxification role in activated marine teleost spermatozoa. The SW-activated trafficking of Aqp8b to the inner mitochondrial membrane facilitates ROS efflux and thus the maintenance of $\Delta \Psi_m$ and ATP production, which are essential for flagellar motility under hypertonic conditions. Although a previous study has shown H$_2$O$_2$ channel activity of mitochondrial AQP8 in human hepatic cultured cells, the physiological significance of this mechanism remains unknown. The present study demonstrates the downstream physiological function of an aquaporin orthologue as a mitochondrial peroxiporin. The expression of aquaporins in seabream spermatozoa, including the water-selective Aqp1aa and -1ab, and water, glycerol and urea-
transporting Aqp7 and -10b paralogues is similar to mammalian sperm where there is convincing evidence for the presence of AQP3, -7, -8 and -11. However, although Aqp1ab, -8b and -10b appear to be phosphorylated upon SW activation of sea spermatozoa, only Aqp8b is rapidly inserted into the inner mitochondrial membrane. The immediacy of Aqp8b accumulation in the mitochondrion is consistent with the extremely fast activation of marine teleost sperm, which is thought to occur in a two-stage process. The first involves water efflux upon contact with the hyperosmotic SW, which rapidly creates an expansion of flagellar membranes driving the local activation of strech-activated channels and the stimulation of the axoneme, followed by an increase of the internal ionic concentration and ATPase activation. Our data suggest that it is the second event that coincides with the recruitment of Aqp8b in the seabream spermatozoan mitochondrion.

This study thus shows that one of the antioxidant systems present in the seabream spermatozoa, presumably in addition to ROS scavenging enzymes, is precisely the accumulation of the peroxiporin Aqp8b in the mitochondrion upon SW activation. As in other marine teleosts, most of the energy required for sea sperm motility is obtained from endogenous ATP stores pre-accumulated during spermogenesis, as well as produced de novo by mitochondrialOXPHOS during the motility phase, rather than glycolysis, which probably plays a minor role during this time. Additional evidence that supports this model is our finding that mito-TEMPO, which can also inhibit the glycolytic pathway, did not affect the ATP content or the motility of activated control spermatozoa. However, OXPHOS-produced ATP also generates ROS as a by-product, which are accumulated in mitochondria possibly together with peroxide generated in the cytoplasm as a result of osmotic stress. Our data in the seabream suggest that to avoid oxidative damage and prolong spermatozoan motility, Aqp8b is specifically delivered to the mitochondrion, where it facilitates the efflux of accumulated H2O2. When this pathway is distorted by the alteration of Aqp8b trafficking and/or channel function, increased mitochondrial ROS levels induce the decrease of the ΔΨm and inhibit the production of ATP, leading to the decline of sperm motility. The existence of this mechanism is further supported by the observation that treatment of immunologically impaired spermatozoa with mito-TEMPO restores the ΔΨm and the production of ATP, and fully rescues sperm motility.

Marine fish spermatozoa are considered hypermotile, showing a high velocity and a fast consumption of energy, when compared to mammalian spermatozoa, which may be an adaptation to the short period during which the microyle remains open in fish eggs after contact with SW. However, mammalian spermatozoa acquire a vigorous swimming activity in the vicinity of the ova, the so-called hyperactivation process, which is needed to traverse the zona pellucida and fertilize the egg. Hyperactivation is triggered by a rise in flagellar Ca2+ and requires increased pH and ATP production. Therefore, the process of hyperactivation occurring in mammalian spermatozoa under hypotonic conditions is reminiscent of the high motility of marine fish sperm, and might require the presence of specific mitochondrial detoxification mechanisms. In rodent and human sperm, however, AQP8 is apparently not found in the mitochondria, rather it is specifically localised in the cytoplasmic droplet, where it is suggested to regulate cell volume upon ejaculation in the oviduct. A potential candidate for mitochondrial peroxide transport might be AQP7, since this aquaglyceroporin can potentially facilitate H2O2 transport, as shown for AQP3, and is localised in the mitochondria of human swim-up motile sperm. Thus, the potential role of aquaporins as functional peroxide channels in mammalian spermatozoa, particularly during hyperactivation, is intriguing and merits further investigation.

In summary, the present data provide the first evidence for the physiological role of a mitochondrial aquaporin-mediated ROS-detoxification pathway in male germ cells. This pathway is essential for the prevention of oxidative damage to activated marine teleost spermatozoa, and may have evolved as a selective advantage for

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**Figure 6** Mitochondrial Aqp8b mediates ROS release during OXPHOS in vitro. (a) Production of ATP by mitochondria isolated from SW-activated spermatozoa in the presence of absence of ADP (2.5 mM), oligomycin (2 mM), IgG or Aqp8b-Ab (0.3 μM). (b) ROS accumulation and release during ATP production by isolated mitochondria treated as in (a), with or without antioxidant mito-TEMPO (50 μM), determined with CM-H2DCFDA. (c) JC-1 red:green fluorescence intensity of mitochondria treated as in A during ATP production. (d) Production of ATP in the presence of ADP, with or without addition of mito-TEMPO. Data (mean ± SEM; n = 3–4 separate experiments on different fish) in (a), (c) and (d) with asterisks are significantly different (P < 0.01) different with respect to mitochondria without ADP, or respect to mitochondria treated with ADP and DMSO alone (brackets). In (b), bars with different superscript are significantly different (P < 0.01). Omy, oligomycin; MitoT, mito-TEMPO.
increased sperm competition. These results uncover a previously unknown mechanism in sperm physiology and open additional avenues for understanding the impact of oxidative stress on fertilisation.

**Methods**

**Animals and sperm collection.** Adult seabream males raised in captivity were maintained as previously described. Milt was recovered from the gonopore of fish sedated with 500 ppm of phenoxyethanol, avoiding SW or urine contamination, stored at 4°C, and used within 24–48 h after collection. Fish samplings were carried out in accordance with the protocols approved by the Ethics Committee of the Institut de Recerca i Tecnologia Agroalimentàries (IRTA, Spain) following the European Union Council Guidelines (86/609/UE).

**Fluorescent probes and antibodies.** The dyes CM-H2DCFDA (C6827), JC-1 (T-3168) and MitoTracker Red CMXRos (M-7512) were from Life Technologies Corp. The Aqp8b-Ab and other affinity-purified antisera against seabream Aqp1aa, -1ab, -7, and -10b have been characterised elsewhere. Antibodies for Hk1, Phb and Hsp60 were from GeneTex (GTX124425, GTX124491 and GTX110039, respectively). Anti-α-tubulin was from Sigma-Aldrich (T9026).

**Aquaporin-mediated water and H2O2 transport in X. laevis oocytes.** Ectopic expression of full-length seabream aquaporin cDNAs in oocytes and determination of the Pf was carried out as described previously. Milt was recovered from the gonopore of fish sedated with 500 ppm of phenoxyethanol, avoiding SW or urine contamination, stored at 4°C, and used within 24–48 h after collection. Fish samplings were carried out in accordance with the protocols approved by the Ethics Committee of the Institut de Recerca i Tecnologia Agroalimentàries (IRTA, Spain) following the European Union Council Guidelines (86/609/UE).

**Sperm-motility assays.** Freshly collected sperm was diluted in NAM (in mg/ml: 3.5 NaCl, 0.11 KCl, 1.23 MgCl2, 0.39 CaCl2, 1.68 NaHCO3, 0.08 glucose, 1 BSA, pH 7.7; 280 mOsm) to 10⁹ cells/mL. Sperm motility and different parameters of sperm movement after 1:10 dilution in filtered SW for 3 min were determined at room temperature by computer-assisted sperm analysis (CASA) using the Integrated Semen Analysis System software (ISASv1, Proiser) as previously described. For antibody inhibition, immotile sperm was incubated in NAM with 0.5% DMSO for 1 h before the assays.

**Biochemical fractionation of spermatozoa.** Sperm mitochondria were isolated following previous protocols slightly modified. Briefly, activated and non-activated sperm (10⁹–10¹⁰ cells/ml) were centrifuged at 1000×g during 1 min and the pellet sonicated for 30 min at 18°C. Oocytes were washed again and the CM-H2DCFDA fluorescence of each well (oocyte + MBS) was measured at excitation and emission wavelengths of 495 nm and 525 nm, respectively, using a multiwell plate reader (Infinite M200, Tecan). Data were background corrected to remove non-specific signals of the MBS alone and of oocytes not exposed to the dye. Mercurial inhibition of water and H2O2 transport was investigated by incubating oocytes in MBS + 100 μM HgCl2 for 15 min before and during the assays. Aquaporin antibodies and rabbit IgG were tested at 0.003–0.3 μM in the presence of 0.5% DMSO for 1 h before the assays.
containing 0.6% digitonin and incubated on ice for 15 min. The extract was diluted 3-fold in 0.3 M sucrose and kept for 15 min on ice before centrifugation at 15000 × g (10 min, 4 °C). The pellet was resuspended in 300 μL IB, sonicated, diluted with 300 μL of 0.3 M sucrose, and centrifuged as above. The pellet was finally resuspended in 10 μL of 0.3 M sucrose and centrifuged at 10000 × g for 1 h, washed, and centrifuged again at 100000 × g for 1 h.

Mitochondrial H2O2 uptake. Mitochondria were resuspended in 10 mL IB + 0.5% DMSO + 200 μM CM-H2DCFDA for 1 h, with or without 0.3 μM of IgG or Aqp8b-Ab, or HgCl2 (10 μM; only during the last 15 min), and centrifuged at 7000 × g for 5 min at 4 °C. Samples were resuspended in equal volumes of fresh IB containing H2O2 (0–300 μM) + 0.5% DMSO in the presence of 0.3 μM IgG or Aqp8b-Ab, or HgCl2 (10 μM), as described above. After 30 min, mitochondria were centrifuged, resuspended in fresh IB, and dispersed in triplicate in 96-well plates. The fluorescence was measured as indicated previously, and the data were background corrected and normalized to the protein concentration determined by a BCA assay.

ATP measurement. ATP content was determined in the whole sperm and mitochondria of non-activated and activated spermatozoa for ~1 min using a bioluminescence ATP determination kit (Life Technologies Corp., A22066). Sperm and mitochondria were lysed in 50 μl of 2% trichloroacetic acid (TCA) + 2 mM EDTA. After strong vortex, 50 μl of the reaction buffer were added to the homogenates. The samples were vortexed again and centrifuged at 14000 × g for 5 min at 4 °C. Duplicated 10-μl aliquots of the supernatant were used for ATP measurement following the manufacturer’s instructions and using a Orion II microplate luminometer (TiterTek-Beethold). Data were normalized to the number of cells or the protein content.

Determination of ROS levels in spermatozoa. Sperm in NAM was loaded with 200 μM of the CM-H2DCFDA dye for 1 h, during which antibodies and drugs were added as described above, centrifuged 1 min at 10000 × g and resuspended in fresh NAM to wash out the dye. Triplicates of 10 μl were loaded in 96-well plates and diluted 1: 10 in NAM or SW prior to measurement of fluorescence intensity. The background signal of NAM or SW sperm without the dye was subtracted to each value.

Membrane-potential assays. Sperm samples in NAM were loaded with the JC-1 probe (1 μg/ml) for 30 min, centrifuged and resuspended either in NAM or SW. Fluorescence intensity was measured for an excitation wavelength of 490 nm and emission wavelengths of 535 nm (green) and 595 nm (red), corresponding to the fluorescence peak of the JC-1 monomer (low ΔΨm) and that of the multimeric aggregates (high ΔΨm), respectively. For the calculation of the red/green fluorescence intensity ratio the background fluorescence intensity of samples not incubated with JC-1 was subtracted.

Mitochondrial OXPHOS in vitro. Mitochondria isolated from SW spermatozoa were incubated for 1 h in IB containing DMSO, IgG or Aqp8b-Ab, with or without mito-TEMPO (as described above for sperm), or with oligomycin (2 mM) for 10 min. Mitochondria were centrifuged at 7000 × g for 5 min, and resuspended in 50 μl of fresh IB supplemented with 1 mM pyruvate and 1 mM malate, with or without 2.5 mM ADP for 1 min. Samples were centrifuged at 8000 × g for 30 s at 4 °C, and the pellet lysed in TCA for ATP measurement. Mitochondrial ROS generation and changes in the ΔΨm were determined with the JC-1/CM-DCFDA and JC-1 reagents, respectively, after 1 min of ATP production as previously described. For ROS detection, the fluorescence intensity of the supernatant (ROS released) and of the pellet resuspended in an equal volume of IS (ROS accumulated) were measured separately and normalized to the protein content.

Protein extraction, antibody precipitation and immunoblotting. Total and plasma membrane fractions of X. laevis oocytes were isolated as described previously. Sperm, mitochondria, and inner mitochondrial membranes were homogenised in 2x Laemmli sample buffer supplemented with protease (EDTA-free Protease Inhibitor Cocktail, Roche) and phosphatase inhibitor (0.5 mM NaF, 1 mM β-glycerophosphate, and 1 mM Na2VO3), and immediately heated at 95 °C for 10 min. In some experiments, mitochondria were digested with calf intestine alkaline phosphatase (New England Biolabs Inc.) for 1 h at 37 °C prior to Laemmli sample buffer addition. For co-precipitation experiments, samples treated with IgG or Aqp8b-Ab were homogenized in 50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1 mM EDTA, 5 mM MgCl2, 1 mM NaF, 1 mM Na2VO3, 0.5% Triton X-100, and protease inhibitors. After separation of ~10% of the homogenate (input), the rest was mixed with Pure ProteomeTM Protein G Magnetic Beads (Merck Millipore) overnight at 4 °C, washed three times with PBS + 0.1% Tween-20, and resuspended in Laemmli sample buffer. Protein electrophoresis (12% SDS-PAGE) and immunoblotting were performed as previously described. Primary antibodies were diluted 1: 1500 (Aqp8b) or 1: 1000 (Hsp60, Phb and Hkl1). For quantification of mitochondrial Aqp8b abundance normalized to that of Hsp60, the intensity of the immunoreactive bands was determined by densitometry using the Quantity-One software (Bio-Rad Laboratories Inc.).

Fluorescence microscopy. Immunofluorescence microscopy on fixed spermatozoa previously labelled with Mitotracker was carried out as described. Antibodies for Aqp8b and α-tubulin were used at 1: 600 and 1: 1000 dilution, respectively. In some experiments, 1: 100 diluted Aqp8b antibodies labelled with Alexa Fluor 488 using the Zenon® Tricolor Rabbit IgG Labeling Kit #1 (Life Technologies Corp., Z-23560) were used. Live spermatozoa labelled with the nucleic acid stain Hoechst 33342 (Life Technologies Corp., H3570) and Mitotracker together with CM-H2DCFDA or JC-1 were immobilised on UltraStick/UltraFrost Adhesion slides (Electron Microscopy Sciences). Images were acquired with a Zeiss Axio Imager Z1/ApO/1.4 fluorescent microscope (Carl Zeiss Corp.) using the same fluorescence intensity and exposure for nonactivated and activated spermatozoa.

Statistics. Results are expressed as the means ± SEM. Comparisons between groups were made by the Student t test or by one- or two-way ANOVA, followed by Tukey’s pairwise comparison. Time-course curves of sperm motility parameters were compared by the Mann-Whitney U test or by one- or two-way ANOVA and the Statgraphics Plus 4.1 software (Statistical Graphics Corp.). P < 0.05 was considered as statistically significant. For all experiments, sperm samples from at least three different males (one ejaculated per male) were used.
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