Maladaptation of trout spermatozoa to fresh water is related to oxidative stress and proteome changes

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Abstract

Rainbow trout sperm are 'maladapted' to freshwater spawning, resulting in shorter duration of sperm motility in fresh water compared to buffered saline solution. We hypothesized that different sperm motility-activating media have various effects on sperm motility characteristics and oxidative stress, as well as on the protein profiles of rainbow trout sperm. We designed an experimental model for activation of rainbow trout sperm motility in different osmotic conditions: (i) isosmotic and (ii) hypoosmotic. Spermatozoa activation with hypoosmotic solution was associated with lower values for sperm motility parameters (52%) and an induced increase in ROS level (19.4%) in comparison to isosmotic activation with isosmotic solution (67 and 9.5% for sperm motility and ROS, respectively). Hypoosmotic activation resulted in a higher number of differentially abundant sperm proteins (out of which 50 were identified) compared to isosmotic conditions, where only two spots of protein disulfide-isomerase 6 were changed in abundance. The proteins are mainly involved in the TCA cycle, tight and gap junction signaling, Sertoli cell–Sertoli cell junction signaling and asparagine degradation. Our results, for the first time, indicate that during hypoosmotic activation of sperm motility, osmotic stress triggers oxidative stress and disturbances mostly to structural proteins and metabolic enzymes. Our results strongly suggest that comparative physiological and biochemical analysis of rainbow trout sperm characteristics in isosmotic and hypoosmotic conditions could be a useful model for studying the mechanism of sperm activation in salmonid fish.

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Introduction

In teleost fish, spermatozoa remain quiescent in the testes and efferent ducts, and activation of motility is induced by changes in the ionic and osmotic aquatic environment into which the sperm are ejaculated (Morisawa & Suzuki 1980). Sperm of teleost fish in general have a short period of motility (30 s to 5 min) and commonly the swimming duration of sperm from marine fish is longer than that of sperm from freshwater fish (Billard & Cosson 1992). Since fish spermatozoa are motile for a very short time, the initiation and activation of sperm motility must be precisely controlled in order to prevent energy exhaustion prior to ejaculation. Sperm motility is acquired under the control of many external and internal factors and is based on the specialized structure of the sperm flagellum. Fish spermatozoa show species differences in the initiation, duration and pattern of motility (Billard & Cosson 1992). In salmonids, the reduction of the external K^+ concentration, upon dilution of semen in water, initiates sperm motility, while in freshwater cyprinids, flagellar motility is initiated by hypoosmotic shock (Morisawa & Suzuki 1980, Stoss 1983, Morisawa 1985). A decrease in environmental K^+ concentration causes K^+ efflux

through specific membrane channels, leading to membrane hyperpolarization which in turn determines the Ca^{2+} influx by calcium channels. An extremely rapid transition of spermatozoa from an immotile to motile state occurs, which is a necessary condition for external fertilization in the running water of streams.

The maladaptation of fish, including trout, spermatozoa to fresh water was recognized a long time ago by Huxley (1930) who indicated that the physiology of trout spermatozoa is imperfectly adapted to fresh water. This suggestion is supported by numerous papers indicating better and longer swimming of spermatozoa in buffered saline solution (Alavi & Cosson 2006) compared to fresh water. Poor resistance of spermatozoa to hypoosmotic shock (Billard & Cosson 1990) is considered to be the one of the limiting factors for the duration of spermatozoan motility in freshwater fish. Since a very short window of sperm motility (Kime *et al.* 2001) is found in salmonids (less than 30 s), buffered saline solution was developed to secure good activation of spermatozoa without exposing them to extreme osmotic conditions, resulting in prolonged sperm motility and thus a higher chance of successful fertilization (Billard 1978). The mechanism related to activation of sperm motility in isosmotic and hypoosmotic conditions is not fully understood;

most studies concern morphological changes of sperm and focus on examining alterations of the structural components of sperm flagella and sperm energetics (Dzyuba & Cosson 2014, Dzyuba *et al.* 2017).

The motility criteria used to characterize fish sperm movement and sperm flagella are well described; however, changes in the protein profiles of sperm as a consequence of motility triggering have been examined only for a few fish species. A proteomic approach was introduced to study the activation of sperm motility in saltwater fish such as gilthead and striped sea bream (Zilli *et al.* 2008) and freshwater common carp and sterlet (Dietrich *et al.* 2016, Gazo *et al.* 2017). The study of Dietrich *et al.* (2016) revealed that activation of carp sperm motility is associated with profound changes to the sperm proteome; 72 differentially expressed proteins were identified, mainly involved in ubiquitin–proteasome pathways, glycolysis, the tricarboxylic acid (TCA) cycle and remodeling and are putatively related to sperm energy metabolism and motility. However, those authors examined the changes in carp sperm proteome during activation only in hypoosmotic conditions in hatchery water. Since it clearly emerges that there are different species-specific mechanisms of sperm motility initiation between cyprinid and salmonid fish, it is unknown at present whether quantitative and/or qualitative protein changes occur during the rainbow trout sperm motility period. To examine this issue, in this study, we designed an experimental model for sperm motility activation of rainbow trout in two different osmotic conditions: (i) isosmotic (Billard solution) and (ii) hypoosmotic (hatchery water).

We formulated a hypothesis that different sperm motility-activating media have various effects on sperm motility characteristics and oxidative stress, as well as on the protein profiles of rainbow trout sperm. We assumed that an isosmotic solution would have a less serious impact on the sperm parameters examined. Therefore, to test this hypothesis, computer-assisted sperm analysis (CASA), flow cytometry and 2D difference gel electrophoresis (2D DIGE) followed by matrix-assisted laser desorption/ionization time-of-flight/time-of-flight mass spectrometry (MALDI-TOF/TOF) identification were employed to monitor changes in sperm parameters and to identify differentially abundant proteins of sperm activated under isosmotic and hypoosmotic conditions.

Materials and methods

Animal sampling

The experiments were carried out on sexually mature spring spawning rainbow trout ($n=7$; 3+ years old; weight = 1254 ± 98 g; total length = 46 ± 2 cm) maintained in the Rutki Salmonid Research Laboratory at the Institute of Inland Fisheries in Olsztyn, Poland. Semen was collected at the beginning of the spawning season. Prior to milt collection,

males were anesthetized using Propiscin (1 ppm; Inland Fisheries Institute, Żabieniec, Poland). Milt from rainbow trout males was obtained by gentle abdominal massage, with special care to avoid contamination with blood, urine or feces. Semen collected from each individual was diluted at a ratio of 1:200 in immobilizing solution for rainbow trout (Morisawa & Morisawa 1988).

Approval by the Animal Experiments Committee in Olsztyn, Poland (no. 39/2011) was obtained before starting the experiments.

Semen analysis

Sperm from the same individual, diluted in immobilizing solution, was divided into two aliquots and activated at a dilution ratio of 1:100 with: (1) 1 mM CaCl_2 , 20 mM Tris, 30 mM glycine and 125 mM NaCl at a pH of 9.0 (Billard 1992) and (2) 1:100 with hatchery water both supplemented with 0.5% bovine serum albumin. The motility parameters of sperm were examined with CASA (Hobson Vision Ltd, Baslow, UK), as described in previous studies (Nynca *et al.* 2014). The following sperm motility parameters were evaluated over a 12-s period, 5–17 s after activation: percentage of motile sperm (MOT), straight-line velocity (VSL), curvilinear velocity (VCL), average path velocity (VAP), linearity (LIN) and the amplitude of lateral head displacement (ALH). Video recordings (two replicates per sample) were obtained using an Olympus BX40 microscope (Olympus Optical) with a 10× negative-phase objective and a Sony CCD black and white camera (SPT-M108CE). Sperm concentration and viability were measured using a Muse Cell Analyzer (Millipore) as described by Nynca *et al.* (2016). The reactive oxygen species (ROS) levels in sperm were estimated using a Muse® Oxidative Stress Kit (EMD Millipore) that simultaneously determines the count and percentage of cells undergoing oxidative stress, based on the intracellular detection of superoxide radicals. The procedure was performed in accordance with the manufacturer's recommendations. To determine accurately the dividing point between ROS^+ and ROS^- populations, a positive control setup was performed by incubating the sperm cells in the presence of 3% H_2O_2 , a superoxide anion generator, for 1 h at RT. Osmolality of seminal plasma was measured using a 5520 Vapor Pressure Osmometer (Wescor, Logan, USA).

Sample preparation

One minute after activation, semen samples were centrifuged at 3000 *g* and 4°C for 10 min. After removing the supernatant, the pellets (containing the spermatozoa) were suspended in lysis buffer containing 7 M urea and 2 M thiourea, 2% CHAPS (3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate hydrate) and sonicated using a VCX-130 Ultrasonic Processor (Sonics & Materials, Inc., Newtown, CT, USA). After incubation for 1 h at 4°C, the samples were centrifuged at 12,000 *g* for 10 min at 4°C. The protein lysates were processed using a Clean-Up Kit (GE Healthcare) according to the manufacturer's protocol. The protein concentration was measured using a Coomassie Plus Kit (Thermo Scientific) with bovine serum albumin as the standard.

2D DIGE analysis and image analysis

Protein labeling with CyDye DIGE Fluor and 2D electrophoresis slices were performed under the same conditions as described by Nynca *et al.* (2015). An aliquot of 50 µg of protein from each sample of (i) immobilized spermatozoa extract, (ii) spermatozoa extract activated by Billard solution and (iii) spermatozoa activated by hatchery water was dissolved in a labeling buffer (7 M urea, 2 M thiourea, 4% w/v CHAPS, 30 mM Tris, pH 8.0) and labeled with CyDye DIGE Fluor minimal dyes (GE Healthcare; reconstituted in fresh 99.8% anhydrous DMF) at a concentration of 400 pmol dye/50 µg of protein. Cy2 was used to label the internal standard, and Cy3 and Cy5 to label individual spermatozoa immobilized and activated by Billard solution and hatchery water sperm samples. A dye swap (Cy3/Cy5) was performed between activated and immobilized sperm samples to exclude dye bias. An internal standard was created by mixing equal amounts of protein from the immobilized and activated spermatozoa. The labeling reaction was performed in the dark on ice for 30 min. Differentially labeled samples (50 µg each of Cy2-, Cy3- and Cy5-labeled samples) were mixed together, uploaded onto 24-cm IPG strips (pH 3–10 NL), and isoelectric focusing was performed with Ettan IPGphor apparatus (GE Healthcare) as described by Nynca *et al.* (2015). As a second dimension, a 12.5% SDS-PAGE (gel size 25.5 × 19.6 cm, 1 mm thickness; GE Healthcare) was run using Ettan Dalt-Six apparatus (GE Healthcare). The CyDye-labeled gels were analyzed by post-run fluorescence imaging with the use of Typhoon FLA 9400 (GE Healthcare) with parameters recommended for 2D DIGE experiments by the manufacturer. After images were acquired, image analysis was performed using DeCyder Differential Analysis software version 5.02 (GE Healthcare). The number of spots was estimated to the nearest 10,000, and a spot volume of 30,000 was used as a cutoff filter. The DeCyder Biological Variation Analysis module was used to detect protein spots and simultaneously match all 12 protein spot maps from six gels. Protein spots with a *P* value <0.05 by *t*-test analysis that showed at least a 1.3-fold increase or decrease in their relative intensity in any comparison of sperm immobilized and activated by Billard solution or hatchery water were considered as differentially expressed. False discovery rate (FDR) correction was applied when the *t*-test was performed. To properly pick and identify the selected spots, DIGE gels were re-stained using Coomassie Brilliant Blue G-250 (Bio-Rad).

Protein digestion and mass spectrometry protein identification

Proteins were digested using a trypsin solution (Promega) containing 0.2 mg/mL trypsin in 15 mM ammonium bicarbonate. Resulting peptide pellets were dissolved in 1 mL of a 5 mg/mL solution of α -cyano-4-hydroxy-cinnamic acid (Bruker Daltonics, Bremen, Germany) in 50% acetonitrile and 0.1% trifluoroacetic acid. MALDI-TOF/TOF analysis was performed using a MALDI TOF tandem mass spectrometer (Autoflex Speed, Bruker Daltonics). The MS together with MS/MS spectra were searched against a NCBI nr Bony Fishes database (a database containing 3,005,095 proteins was

generated on the 30th January 2017 using the Mascot Server (Matrix Science, London, UK) of the National Center for Biotechnology, in consideration of the following settings: (i) enzyme: trypsin; (ii) fixed modifications: carbamidomethyl; (iii) variable modifications: methionine oxidation; (iv) allow one missed cleavage; (v) peptide tol. 150 ppm, MS/MS tol. 0.7 Da; (vi) peptide charge 1+; (vii) instrument type: MALDI-TOF/TOF. The Mascot identity threshold score was defined as 42 for *P* < 0.05 Mascot score. In other words, the protein sequence was identified when at least two individual peptides per protein, each of whose probability scores met or exceeded the threshold for statistical significance (ion score >42, *P* < 0.05).

Ontology and ingenuity pathway analyses of the identified proteins

Gene ontology (GO) annotation was acquired by matching the GI numbers of identified proteins to the UniProtKB database (www.uniprot.org). The category used was 'molecular functions'. Ingenuity Pathway Analysis (IPA; Qiagen Silicon Valley) of the identified proteins used the Core Analysis function to interpret the identified proteins in the context of biological functions and pathways. Because IPA only accepts gene or protein accession numbers representing human, mouse and rat genes or proteins, orthologs of the identified rainbow trout proteins belonging to those three species were first identified, and the accession numbers of the top blast hits were uploaded to IPA. The significance of biological functions and canonical pathways was tested automatically by the software, with use of the Fisher's exact test *P* value. Top significant functions and canonical pathways were selected by the software for each tested proteome. Additionally, the proteins with larger expression (greater than 2-fold change) after hypoosmotic activation were analyzed by g:Profiler (<https://biit.cs.ut.ee/gprofiler/gost>).

Statistical analysis

All the results of the semen parameters are presented as means ± s.d. For statistical procedures, percentage data for sperm motility parameters were transformed by arcsin square-root transformation. The sperm motility parameters data were subjected to a Student's *t*-test for paired samples; data regarding sperm viability and ROS-positive cell content were subjected to one-way ANOVA followed by Tukey's multiple comparisons test. All analyses were performed at a significance level of 0.05 using GraphPad Prism software (GraphPad Software Inc.).

Results

Effect of activation solution on sperm motility parameters, viability and ROS

The two activation solutions affected sperm motility parameters in a different manner (Fig. 1A, B, C, D, E and F). Activation with Billard solution resulted in higher values (*P* ≤ 0.05) for sperm motility parameters

Figure 1 Effect of isosmotic and hypoosmotic activation on sperm motility parameters of rainbow trout ($n=7$). (A) Percentage of sperm motility, (B) VCL (curvilinear line velocity), (C) VSL (straight line velocity), (D) VAP (average path velocity), (E) ALH (amplitude of lateral head displacement), (F) LIN (linearity). Results are expressed as means $\pm$ s.d. Asterisk indicates significant differences between parameters for activated sperm in isosmotic and hypoosmotic conditions (* $P<0.05$, ** $P<0.01$, *** $P<0.001$).

except LIN, for which lower values were noticed in comparison to activation of sperm with hatchery water (Fig. 1F). Different activation solutions used for sperm activation had no effect on sperm viability (Fig. 2A), but significantly ($P\leq 0.05$) increased the proportion of ROS-positive cells, that reached 19.4% for semen activated with H_2O . At the same time, the increase by 9.5% was not significant for the activation with Billard solution (Fig. 2B). The average sperm concentration and

Figure 2 Effect of sperm activation on viability (A) and ROS-positive cell content of rainbow trout semen (B) ($n=7$). Results are expressed as means $\pm$ s.d. Asterisk indicates significant differences between ROS-positive cell content for immobilized and activated sperm in isosmotic and hypoosmotic conditions ($P<0.05$).

osmolality of samples used for the experiment were $11.7 \pm 3.3 \times 10^9/\text{mL}$ and 280 ± 9 mosmol/kg, respectively.

Comparison of immobilized and activated sperm proteome

The analysis of sperm immobilized and activated with hatchery water using 2D DIGE technology led to the detection of 1260 matched spots and 61 spots with a significant difference ($P\leq 0.05$) in their relative abundance between immobilized and activated sperm (Fig. 3A and B, Supplementary Table 1, see section on supplementary data given at the end of this article). Thirty-nine spots were enriched in sperm activated with H_2O , and 22 spots were more abundant in immobilized sperm. Out of 38 spots with a greater abundance in H_2O -activated sperm, 30 spots were identified by MALDI-TOF/TOF, whereas out of 23 spots upregulated in immobilized sperm, 20 spots were identified (Table 1, Supplementary Table 2). Amongst identified proteins 13 proteins have been identified with higher expression, the cAMP-dependent protein kinase type II was the protein which most increased in response to hypoosmotic activation (fold change 3.53), whereas cilia- and flagella-associated protein 58 was the most downregulated (fold change -5.26). The 22 proteins were characterized by small changes in expression (-1.4 and <1.4).

Isosmotic activation led to the detection of two spots with a significant difference in their relative abundance when compared to immobilized sperm (Fig. 3C and D). Those spots (nos. 44 and 47) corresponded to spots with a higher abundance in H_2O -activated sperm (Table 2).

GO annotation and signaling pathway analysis of differentially abundant proteins

The 50 proteins identified with a change in abundance in sperm activated with hatchery water were classified according to the 'molecular function' GO database (Fig. 4). Based on molecular function analysis, proteins were divided into four groups, with the majority having binding and catalytic activity (Fig. 4).

A summary of all associated pathways, annotated using IPA, for proteins differentially abundant in immobilized and activated sperm is provided in Table 3. The top canonical pathways associated with proteins changed during sperm activation include the TCA cycle, tight and gap junction signaling, Sertoli cell–Sertoli cell junction signaling and asparagine degradation, while the molecular and cellular functions include amino acid metabolism, small-molecule biochemistry, cellular function and maintenance, cellular movement, assembly and organization (Table 3). From a list of top networks generated using IPA, for further analysis we selected the most representative network associated with the functions that were more pertinent to the sperm activation such as cellular movement (Fig. 5).

Figure 3 2D DIGE analysis of immobilized and activated rainbow trout spermatozoa. Numbered protein spots (1–61) correspond to proteins identified from 2D DIGE which changed in abundance after activation of immobilized sperm (A) in hypoosmotic conditions (B). Numbered protein spots (44, 47) correspond to proteins identified from 2D DIGE which changed in abundance after activation of immobilized sperm (C) in isosmotic conditions (D). The nonlinear pH range of the first-dimension IPG strip is indicated along the top of the gel, acidic pH to the left. The MW (relative molecular mass) scale can be used to estimate the molecular weights of the separated proteins.

The network with a score of 38, in which 11 proteins show increasing trends expression during activation (EFHC1, EFHC2, TEK2, DNAI2, PRKAR2A, TUBB4B, SPAG6, CCDC40, CABYR, OGDH, ACAT1) and 5 proteins were decreased. Most of the sperm proteins displaying a change in level after motility activation are

closely connected in the network through nodes Ca^{2+} and PRKAR2A being reported to be involved in sperm motility (Itoh *et al.* 2003, Dietrich *et al.* 2016). A majority of proteins were observed to be cytoplasmic in origin. They were involved in functions related to enzyme activity, kinase, peptidase, the G-protein-coupled

Table 1 Proteins that showed a significant change in abundance in activated sperm compared to immobilized sperm of rainbow trout.

No.	Protein	Gene name	NCBI accession	Mass/pI	Score	No unique peptides	Sequence Cov	Fold change	Protein function
1	Cytoskeleton, flagella and cell movement Predicted: coiled-coil domain-containing protein 40 [Salmo salar]	<i>CCDC40</i>	XP_014032980.1	113599/5.92	243	2	23	1.33	Sperm motility, axonemal dynein complex assembly
2	Predicted: coiled-coil domain-containing protein 40 [Salmo salar]	<i>CCDC40</i>	XP_014032980.1	113599/5.92	219	2	20	1.34	Sperm motility, axonemal dynein complex assembly
23	PREDICTED: coiled-coil domain-containing protein 173 [Salmo salar]	<i>CCDC173</i>	gi 642131459	64775/9.16	163	3	25	-1.81	Sperm motility, axonemal dynein complex assembly
26	PREDICTED: cilia- and flagella-associated protein 53 [Salmo salar]	<i>CFAP53</i>	CDQ60305.1	63646/8.35	269	3	31	-1.87	Cilium movement and assembly
29	PREDICTED: coiled-coil domain-containing protein 183 isoform X2 [Salmo salar]	CCDC183	CCDC183	34551/5.76	302	3	57	2.73	Sperm motility, axonemal dynein complex assembly
30	PREDICTED: coiled-coil domain-containing protein 183 isoform X2 [Salmo salar]	<i>CCDC183</i>	gi 642094391	34551/5.76	391	6	54	-1.69	Sperm motility, axonemal dynein complex assembly
60	PREDICTED: coiled-coil domain-containing protein 173 [Salmo salar]	<i>CCDC173</i>	gi 642131459	64775/9.16	272	3	16	1.82	Sperm motility, axonemal dynein complex assembly
8	Cilia- and flagella-associated protein 58 [Salmo salar]	CFAP58	gi 929221592	103858/8.12	577	6	53	-5.26	Cilium movement and assembly
19	PREDICTED: cilia- and flagella-associated protein 45-like [Salmo salar]	CFAP45	XP_014070227.1	68270/8.64	242	3	33	-2.44	Cilium movement and assembly
20	PREDICTED: cilia- and flagella-associated protein 45-like [Salmo salar]	<i>CFAP45</i>	gi 642122481	68270/8.64	338	4	34	-1.76	Cilium movement and assembly
22	PREDICTED: cilia- and flagella-associated protein 45-like [Salmo salar]	CFAP45	gi 642122481	68270/8.64	203	3	24	-2.15	Cilium movement and assembly
25	PREDICTED: cilia- and flagella-associated protein 53 [Salmo salar]	<i>CFAP53</i>	CDQ60305.1	63646/8.35	349	4	33	-1.92	Cilium assembly Cilium movement
9	Dynein regulatory complex protein 1 [Salmo salar]	<i>DRC1</i>	gi 929249040	86743/5.40	253	3	32	-1.87	Axonemal dynein complex assembly
10	PREDICTED: dynein regulatory complex protein 1 [Salmo salar]	DRC1	XP_014000687.1	86743/5.40	208	2	28	2.98	Axonemal dynein complex assembly cilium-dependent cell motility ATP binding, microtubule motor activity
15	Dynein intermediate chain 3, ciliary-like [Salmo salar]	<i>DYNLL2</i>	gi 929056610	67665/5.13	497	6	35	1.30	Cell motility
17	PREDICTED: radial spoke head protein 6 homolog A-like [Salmo salar]	<i>RSPH6A</i>	XP_014051759.1	59724/4.29	324	4	35	1.32	Form part of the axoneme of flagella Regulate the activity of dynein and the symmetry of flagellar bending patterns

(Continued)

Table 1 Continued.

No.	Protein	Gene name	NCBI accession	Mass/pI	Score	No unique peptides	Sequence Cov	Fold change	Protein function
31	Leucine-rich repeat-containing protein 48 isoform X1 [Salmo salar]	DRC3	gi 929095036	61057/4.67	138	3	19	1.30	Regulator of ciliary/flagellar motility which maintains the alignment and integrity of the distal axoneme and regulates microtubule sliding in motile axonemes
4	Predicted: WD repeat-containing protein 63 isoform X2 [Salmo salar]	WDR63	gi 929122470	89254/5.87	338	4	23	2.44	Dynein chain binding
36	PREDICTED: tektin-2 isoform X3	TEKT2	gi 642132208	50824/5.89	424	5	61	1.37	Microtubule-based movement Cilium movement involved in cell motility, cilium assembly
39	PREDICTED: tubulin beta-4B chain-like [Salmo salar]	TUBB4B	XP_014005633.1	50255/ 4.79	186	2	21	2.89	Microtubule-based process
50	Actin, cytoplasmic 2 [Salmo salar]	ACTG1	gi 924442847	42053/5.24	521	5	58	1.35	Cell motility
51	Actin, cytoplasmic 2 [Salmo salar]	ACTG1	gi 642077850	40789/4.95	120	2	15	1.89	Cell motility
55	PREDICTED: outer dense fiber protein 3B [Salmo salar]	RSPH9	XP_013980502.1	28054/10.03	499	6	54	-2.25	Stabilize the axoneme to maintain sperm motility
56	Predicted: radial spoke head protein 9 homolog [O. mykiss]	RSPH9	gi 642131999	31086/5.66	537	5	44	1.32	Cilium movement involved in cell motility Component of the axonemal radial spoke head. Radial spokes are regularly spaced along cilia, sperm and flagella axonemes
57	PREDICTED: outer dense fiber protein 3B [Salmo salar]	ODF3	XP_013980502.1	28054/10.03	563	6	57	-2.41	Outer dense fibers are filamentous structures located on the outside of the axoneme in the midpiece and principal piece of the mammalian sperm tail. May help to maintain the passive elastic structures and elastic recoil of the sperm tail Important for structural integrity of the central apparatus in the sperm tail and for flagellar motility
45	Sperm-associated antigen 6 [O. mykiss]	SPAG6	gi 642089502	58032/6.86	469	6	32	1.35	tricarboxylic acid cycle, glycolytic process Chaperone binding
Metabolism and energy production									
5	2-Oxoglutarate dehydrogenase, mitochondrial isoform X2 [Salmo salar]	OGDH	XP_014030343.1	116248/6.30	239	4	20	1.32	Tricarboxylic acid cycle, glycolytic process Chaperone binding
6	2-Oxoglutarate dehydrogenase, mitochondrial isoform X1 [Salmo salar]	OGDH	gi 929305625	117908/6.30	500	6	28	1.35	Tricarboxylic acid cycle, glycolytic process Chaperone binding
12	Predicted: aconitate hydratase [O. mykiss]	ACO2	gi 642077361	80305/7.29	200	3	14	-1.30	Tricarboxylic acid cycle iron ion binding
33	Glutamate dehydrogenase [Salmo salar]	GLUD1	gi 30314696	60576/8.45	209	3	16	-1.53	ATP binding Tricarboxylic acid metabolic process
34	Glutamate dehydrogenase [Salmo salar]	GLUD1	gi 30314696	60576/8.45	152	3	11	-1.69	ATP binding Tricarboxylic acid metabolic process

18	PREDICTED: propionyl-CoA carboxylase alpha chain, mitochondrial-like [Salmo salar]	PCCA	XP_014040128.1	45800/5.47	101	2	15	2.19	Catalyzes the carboxylation of propionyl-CoA producing methylmalonyl-CoA which is then converted to succinyl-CoA, an intermediate in TCA
16	PREDICTED: methylcrotonoyl-CoA carboxylase subunit alpha, mitochondrial-like [Salmo salar]	MCCC1	XP_014024859.1	78442/5.79	116	3	12	1.32	ATP binding Amino acid metabolism ATP binding Metal ion binding Biotin-attachment subunit of the 3-methylcrotonyl-CoA carboxylase, an enzyme that catalyzes the conversion of 3-methylcrotonyl-CoA to 3-methylglutaconyl-CoA, a critical step for leucine and isovaleric acid catabolism
35	Methylmalonate-semialdehyde dehydrogenase [Salmo salar]	ALDH6A1	gi 218505653	57313/6.48	195	2	19	1.31	Plays a role in valine and pyrimidine metabolism. Binds fatty acyl-CoA
37	PREDICTED: adenylate kinase X1 [Salmo salar]	AK8	CDQ66442.1	54510/5.80	414	5	35	1.38	Fatty-acyl-CoA binding ATP binding adenylate Kinase activity Nucleoside diphosphate kinase activity
48	Creatine kinase, testis isozyme [O. mykiss]	CKB	gi 185132569	43262/6.20	416	5	49	-1.46	ATP binding Phosphocreatine biosynthetic process
52	Acetyl-CoA acetyltransferase, mitochondrial [Salmo salar]	ACAT1	gi 929084622	44399/9.03	545	5	44	1.32	Fatty acid beta-oxidation
13	EF-hand domain-containing family member C2 isoform X2 [Salmo salar]	EFHC2	gi 929285878	86530/6.35	276	4	28	1.32	Calcium ion binding
14	EF-hand domain-containing family member C2 isoform X2 [Salmo salar]	EFHC2	gi 929285878	86530/6.35	453	4	42	1.37	Calcium ion binding
59	PREDICTED: EF-hand domain-containing protein 1 isoform X1 [Salmo salar]	EFHC1	XP_013999371.1	65064/6.07	314	3	29	-1.52	Alpha-tubulin binding Calcium ion binding Microtubule-associated protein which regulates cell division
3	Uncharacterized protein LOC106584254 isoform X1 [Salmo salar]	CABYR	XP_014024777.1	89043/5.63	168	3	10	1.91	Calcium ion binding Sperm capacitation
40	PREDICTED: cAMP-dependent protein kinase type II-alpha regulatory subunit-like isoform X3 [O. mykiss]	PRKAR2A	gi 642078503	42170/4.89	332	4	41	-2.51	Intracellular signal transduction Activation of protein kinase A activity, regulatory subunit of the cAMP-dependent protein kinases involved in cAMP signaling in cells
46	PREDICTED: cAMP-dependent protein kinase type II-alpha regulatory subunit-like isoform X3 [O. mykiss]	PRKAR2A	gi 642078503	42170/4.89	275	3	49	3.53	Intracellular signal transduction Activation of protein kinase A activity, regulatory subunit of the cAMP-dependent protein kinases involved in cAMP signaling in cells

(Continued)

Table 1 Continued.

No.	Protein	Gene name	NCBI accession	Mass/pI	Score	No unique peptides	Sequence Cov	Fold change	Protein function
53	Serine/threonine-protein phosphatase 2A catalytic subunit beta [<i>Clupea harengus</i>]	<i>PPP2CB</i>	XP_012687205.1	36273/5.22	194	3	31	1.69	Signal transduction Metal ion binding Protein dephosphorylation Apoptotic mitochondrial changes
Protein folding and turnover									
44	PREDICTED: protein disulfide-isomerase A6-like [O. mykiss]	<i>PDI/A6</i>	CDQ62935.1	48237/5.21	155	2	24	1.30	Protein folding, cell redox homeostasis
47	Protein disulfide-isomerase A6-like [O. mykiss]	<i>PDI/A6</i>	gi 929098196	48229/4.88	125	2	12	-1.31	Protein folding, cell redox homeostasis
Oxidation-reduction process									
61	Thioredoxin domain-containing protein 12 precursor [<i>Salmo salar</i>]	<i>TXNDC12</i>	NP_001139851.1	19247/5.12	293	4	33	1.37	Redox regulation, defense against oxidative stress Peptide disulfide oxidoreductase activity Cell redox homeostasis
Other pathways									
42	PREDICTED: uncharacterized protein LOC105935932 [<i>Fundulus heteroclitus</i>]	<i>HIST2H3A</i>	XP_012732015.1	43965/11.41	359	6	26	1.68	
54	Predicted: protein FAM166B [O. mykiss]	FAM166B	gi 642128870	33157/9.78	615	7	78	-2.99	
58	L-Asparaginase-like [<i>Salmo salar</i>]	<i>ASPG</i>	gi 929085371	29538/4.98	253	3	36	1.32	Asparagine metabolic process

Shadow boxes indicate proteins with larger changes in protein expression ($-2>$ and >2).

receptor, growth factor and transcription regulator. The proteins with larger expression after hypoosmotic activation were analyzed by g:Profiler in terms of their biological process and cellular component. Axoneme assembly and microtubule formation were found as top biological process.

Discussion

This is the first comparative study of the impact of different activation media (isosmotic, hypoosmotic) on rainbow trout sperm characteristics including sperm motility parameters, viability, oxidative stress and protein profile. Spermatozoa activation with hatchery water was associated with lower values for sperm motility parameters and an induced increase in ROS level in comparison to Billard solution activation. The application of a 2D DIGE approach revealed a higher number of differentially abundant sperm proteins after activation with hatchery water and facilitated the identification of 50 protein spots. On the other hand, activation with Billard solution led to the identification of only two spots changed in response to activation which were also changed after activation with hatchery water. The 50 differentially abundant proteins in activated sperm were involved in a variety of pathways including the TCA cycle, tight and gap junction signaling, Sertoli cell–Sertoli cell junction signaling and asparagine degradation.

Motility parameters and model

In externally fertilizing species, osmolality of the fertilization medium is known to play a critical role in activating sperm motility; exposure to a hypoosmotic environment triggers the initiation of salmonid fish sperm motility (Alavi & Cosson 2006). In our study, osmolality of the activation medium (Billard solution, hatchery water) clearly affected the sperm motility parameters in a different manner. Sperm motility activated with Billard solution resulted in mostly higher values of CASA parameters compared to activation of sperm with hatchery water. These results clearly demonstrate the different action of osmolality on spermatozoa motility, since Billard solution is isosmotic to seminal plasma (approximately 300 mosmol/kg), whereas H₂O is hypoosmotic (low osmolality medium, below 50 mosmol/kg). Summing up, the CASA analysis results allowed us to clearly distinguish two modes of sperm activation in rainbow trout and verified our experimental model which was a prerequisite for our study.

Viability is not affected by osmotic stress

Under the conditions of our experimental design, we did not observe significant changes in viability. It is well known that low osmolality induces sperm cell swelling,

Table 2 Proteins that showed a significant change in abundance in isosmotic activated sperm compared to immobilized sperm of rainbow trout.

No.	Protein	Gene name	NCBI accession	Mass/pI	Score	No unique peptides	Sequence Cov	Fold change	Protein function
44	PREDICTED: protein disulfide-isomerase A6-like [O. mykiss]	PDIA6	CDQ62935.1	48237/5.21	155	2	22	1.43	Protein folding, cell redox homeostasis
47	Protein disulfide-isomerase A6-like [O. mykiss]	PDIA6	gi 929098196	48229/4.88	125	2	14	−1.51	Protein folding, cell redox homeostasis

and disruption of the plasma membrane occurs due to the large osmotic shock (Morisawa *et al.* 1983). However, in our study, serious disruption of spermatozoa likely did not occur, because different osmolality of the activating medium led to the activation of rainbow trout sperm motility, but without affecting the viability of spermatozoa. It is well known that the application of activating isosmotic media during artificial fertilization of salmonids leads to higher fertilization rate (Billard 1992) compared to hypoosmotic hatchery water. The dilution of trout sperm in a saline solution (Billard solution) resulted in about 80% fertilization rate in comparison to dilution in fresh water where about 60% of fertilization was achieved (Billard 1990). These solutions not only positively affect spermatozoa motility and prolong the time during which eggs can be fertilized due to reduced osmotic shock but also prevent the harmful effects of pollution by yolk released from broken oocytes which might block the micropyle of eggs (Billard 1992).

Osmotic stress causes oxidative stress

In our study, we have observed a clear difference between sperm activated in water and in Billard solution regarding ROS-positive cell content. Our results indicate that sperm activation with hatchery water causes an increase in ROS production and induces greater changes in sperm protein profile (see below) compared to Billard activation. This strongly suggests

that osmotic stress induces an oxidative stress in fish spermatozoa activated with water. Such a phenomenon has been described for mammals (Burnaugh *et al.* 2010, McCarthy *et al.* 2010), and recently the phenomenon has also been suggested for fish. Dietrich *et al.* (2016) recorded changes in oxidative proteins (peroxiredoxin and glutathione-S-transferase) during activation of carp sperm motility in water. This suggests that antioxidant enzymes play a vital role in coping with oxidative stress caused by osmotic stress. Dzyuba *et al.* (2016) recorded higher levels of thiobarbituric acid reactive substances (TBARS, an indicator of lipid peroxidation) in tench spermatozoa activated at 0mosmol/kg compared to non-activated sperm. These authors also observed an increase in TBARS due to activation at 100mosmol/kg, although this increase was not significant. In our study, we did not observe ROS production in sperm activated with Billard solution which is isosmotic for fish. Altogether, our results and those of Dietrich *et al.* (2016) and Dzyuba *et al.* (2016) collectively suggest that, in fish, osmotic stress which triggers activation of sperm motility causes oxidative stress at the same time. More detailed further studies are necessary to test the kinetics between osmotic stress and oxidative stress in order to unravel the links between the mechanisms of osmotic and oxidative stress.

Superoxide anion seems not to be important in activation of sperm motility in rainbow trout

Our results strongly suggest that, contrary to hypotonic activation, isosmotic activation of sperm motility in rainbow trout is not accompanied by substantial oxidative stress caused by ROS, because no significant increase in ROS-positive spermatozoa was observed in spermatozoa activated with Billard’s solution. This result was not expected in view of the postulated role of ROS in sperm activation, including in fish (de Lamirande & O’Flaherty 2008, Dzyuba & Cosson 2014, Pereira *et al.* 2017). However, it should be stressed that the Muse Oxidative Stress Kit is based on the use of dihydroethidium (DHE), a well-characterized reagent that has been extensively used to detect ROS in cellular populations (Anonymous 2013). This reagent is specific for superoxide anion but no other oxidizing species such as H₂O₂, hypochlorous acid or peroxynitrite (Fink *et al.* 2004). Therefore, it can

Figure 4 Gene ontology annotations for rainbow trout sperm proteins according to the GO term ‘molecular function’.

Table 3 Functional analysis of sperm proteins changed in response to activation in hypoosmotic conditions.

Top canonical pathways	P-Value*	Molecules	Molecular and cellular functions	P-Value*	Top networks	Score**
TCA cycle	6.45E-04	OGDH, ACO2	Amino acid metabolism	2.91E-02–1.05E-07	Cellular movement, reproductive system development and function, cellular function and maintenance	38
Tight junction signaling	2.55E-03	ACTG1, PPP2CB, PRKAR2A	Small molecule biochemistry	2.91E-02–1.05E-07	Post-translational modification	27
Gap junction signaling	2.73E-03	ACTG1, TUBB4B, PRKAR2A	Cellular function and maintenance	1.14E-02–2.54E-05	Molecular transport	7
Sertoli cell–Sertoli Cell junction signaling	3.06E-03	ACTG1, TUBB4B, PRKAR2A	Cellular movement	9.68E-04–2.54E-05		
Asparagine degradation I	3.27E-03	ASPG	Cellular assembly and organization	2.27E-02–5.09E-04		

*The *P*-value associated with a function or a pathway is a measure of the likelihood that the association between a set of focus genes in the experiment and a given process or pathway is due to random chance. The smaller the *P*-value the less likely that the association is random and the more significant the association. In general, a *P* value (calculated using the right-tailed Fisher exact test) <0.05 indicates a statistically significant, nonrandom association. **The score is derived from a *P*-value and indicates the likelihood of the Focus Genes in a network being found together due to random chance. The score takes into account the number of focus genes in the network and the size of the network to approximate how relevant this network is to the original list of focus genes.

ACO2, aconitate hydratase; ACTG1, actin, cytoplasmic 2; ASPG, L-asparaginase; OGDH, 2-oxoglutarate dehydrogenase; PP2CB, serine/threonine-protein phosphatase 2A catalytic subunit beta; PRKAR2A, cAMP-dependent protein kinase type II-alpha; TUBB4B, tubulin beta-4B.

be suggested that superoxide anion is not important in the activation of sperm motility in trout. Perhaps other reactive species are more important for the activation of sperm motility, such as peroxynitrite and its precursor nitric oxide. The latter has been postulated to be involved in sperm motility of fathead minnow (Creech *et al.* 1998), catfish (Barman *et al.* 2013) and steelhead (Wilson-Leedy & Ingermann 2011).

Only a single protein changed after isosmotic activation

Remarkably, contrary to hypotonic activation, only one protein responded to the activation in isosmotic conditions – protein disulfide-isomerase 6 (PDI6). This finding was not expected because we rather anticipated changes in proteins involved in the sperm motility apparatus and sperm metabolism which are the most important characteristics of motile spermatozoa. PDI is involved in correct folding of the proteins through oxidation, reduction and isomerization of disulfide bonds. PDI is a member of the thioredoxin superfamily and can act as an oxidant by forming intra-chain disulfide bonds, which leads to the acquisition of proper conformation by proteins (Yoshioka 2015). To fulfill this function, PDI occurs either in an oxidized or reduced state. In human spermatozoa, PDI6 is located in the peri-acrosomal region, has been associated with chaperone activity and capacitation (Bromfield *et al.* 2016) and has been postulated as a fertility marker (Schorr-Lenz *et al.* 2016). No evidence for the possible mechanisms of PDI6 action in fish sperm is available but it seems to be clear that involvement in cell signaling has to be part of such a mechanism. This suggestion is supported by the involvement of PDI in numerous redox cell-signaling actions through regulation of

thiol–disulfide switches, including those on the cell surface (Laurindo *et al.* 2012). Most importantly, it has been found that peroxynitrite can oxidize and specifically regulate PDI (Peixoto *et al.* 2018). This agrees with the postulated role of peroxynitrite in the activation of sperm movement (see section on Superoxide anion seems not to be important in activation of sperm motility in rainbow trout). PDI has also been found to be a target for protein S-nitrosylation in human spermatozoa (Lefièvre *et al.* 2007). Altogether, the information presented clearly demonstrates the involvement of PDI in the signaling mechanisms of the cells. This opens an exciting area of research aimed at testing the possible involvement of PDI in activation of sperm motility in salmonid fish, and possibly in other fish species as well, in view of identification of PDI during activation of carp spermatozoa (Dietrich *et al.* 2016).

The exact mechanism concerning an increase of PDI abundance in response to activation of sperm motility is unknown at present. Obviously, PDI biosynthesis must be ruled out because of the extremely short time of activation. Moreover, translation in spermatozoa is absent or extremely restricted due to compaction of the haploid genome. It is likely that changes in PDI conformation are rather related to post-translational modifications (PTMs). Indeed, several PTMs have been reported for PDI, including S-nitrosylation, glutathionylation, phosphorylation, arginylation, cleavage, 4-hydroxynonenal addition, O-linked N-acetylglucosamine addition and metal binding (Laurindo *et al.* 2012). Identification of specific PTMs in rainbow trout PDI and the precise functions of such modifications need to be validated in further studies, which may be critical for better understanding of sperm motility activation in salmonid fish.

Figure 5 Cellular movement as signaling pathways associated with rainbow trout sperm motility activation. Red denotes proteins that increased after sperm motility activation, while green denotes proteins that decreased after sperm motility activation. Color intensity indicates expression level. The proteins filled in 'red' and 'green' are the ones identified in our study while the remaining ones are the proteins in the Ingenuity Knowledge Base that interact with ours (unfilled were added by IPA software to complete the pathway). Lines indicate interactions, with the arrowheads indicating directionality. Absence of arrowheads refers to a binding interaction. Dotted line indicates an inferred or indirect interaction. Nodes are represented with various shapes to distinguish the functional class of the molecule: $\diamond$ enzyme, $\square$ G-protein coupled receptor, $\square$ growth factor, $\blacktriangledown$ kinase, $\diamond$ peptidase, transcription regulator, $\bigcirc$ group or complex, $\bigcirc$ other.

Greater changes in protein abundance after hypoosmotic activation

In our study, after activation with hatchery water, the sperm proteome changed to a greater extent in comparison to Billard solution activation. Extensive changes in protein abundance of sperm after hatchery water activation are likely to be the results of osmotic and oxidative stress. Osmotic stress leads to damage

Figure 6 Substructures comprising sperm flagellar axoneme with marked identified proteins changed after activation in hypoosmotic conditions. Proteins that show marked changes (≥ 2 and ≤ -2) in expression are shown in red. Proteins that show minor changes (< 2 and > -2) in expression are shown in green.

of the plasma membrane and alteration in sperm metabolism. ROS can damage cell structures such as carbohydrates, nucleic acids, lipids and proteins and alter their functions (Birben *et al.* 2012). Subsequent alterations in protein conformation can lead to increased aggregation, fragmentation, distortion of secondary and tertiary structure, and susceptibility to proteolysis and diminution of normal function (Butterfield *et al.* 1998). In turn, oxidative stress may lead to alterations in protein expression levels in spermatozoa, causing molecular and genetic defects and impairing male fertility (Morielli & O'Flaherty 2015). In our study, the decrease in protein abundance after motility activation with water could also be the result of leakage of proteins from spermatozoa. On the other hand, an increase in protein abundance could be linked with PTMs and activation of proproteins by restricted proteolysis, as was postulated for mammalian spermatozoa (Kwon *et al.* 2014, Westfalewicz *et al.* 2015). In our study the most extensive changes in protein abundance after hypoosmotic activation were found for cAMP-dependent protein kinase (fold change 3.53) and for cilia- and flagella-associated protein 58 (fold change -5.26). Those proteins are involved in sperm motility. The cyclic AMP-dependent phosphorylation of proteins is essential for the initiation of sperm motility in salmonid fishes, whereas cilium- and flagellum-specific proteins play a role in axonemal structure organization and motility.

Hypoosmotic activation affected mostly proteins related to cytoskeleton, flagella and cell movement

A plethora of sperm proteins changed after hatchery water activation were found to be associated with cell movement. Their potential localization is shown in Fig. 6. These proteins are constituents of several structures of flagella including outer and inner dynein arms, microtubules, radial spoke, central apparatus, dynein regulatory complex and other axonemal structures and contribute to the stability of spermatozoa and formation of flagella, and therefore, play an important role in sperm motility (Baltz *et al.* 1990, Inaba *et al.* 1998). For example, coiled-coil domain proteins interact with the motor protein kinesin, which in turn participates in axoneme formation in the sperm flagellum (Lehti *et al.* 2013). Tektins are involved in the formation of sperm flagella; they are necessary for the proper coordinated beating of the sperm flagellum and are required for progressive sperm motility (Roy *et al.* 2009). Actin and tubulin participate in flagellar motility by dynein-driven microtubule sliding. In turn, dynein regulatory proteins are involved not only in the structural maintenance of flagellar elasticity but also play a regulatory role in flagellar motility (Si & Okuno 1993). Identification of tubulin and dynein is in agreement with data obtained by Dietrich *et al.* (2016) for common carp activation in hypoosmotic conditions. Taken together, the activation of sperm motility in rainbow trout and possibly other freshwater fish is associated with profound changes in the structural components of sperm flagella. The number of significant changes in the sperm proteome related to the cytoskeleton, flagella and cell movement strongly suggest dynamic changes in the sperm motility apparatus in response to activation of movement in hypoosmotic conditions. On the other hand, some of these changes could reflect negative effects related to osmotic stress, which results in lower quality of movement compared to activation in isosmotic conditions.

Hypoosmotic activation affects proteins associated with metabolism

The changes occurring within the proteome observed after sperm motility activation in hypoosmotic conditions are associated with changes in proteins related to metabolism and energy production. Categorization of proteins according to molecular function indicates that the majority of proteins are involved in catalytic activity and binding. In this study, we identified spermatozoa proteins involved in glycolysis, the TCA cycle (indicated as a top canonical pathway by IPA), the fatty acid cycle, amino acid metabolism (indicated as a key molecular and cellular function by IPA) and ATP binding. Those results agree with previous findings indicating the importance of lipid metabolism and glycolysis for energy

provision in sperm (Lahnsteiner *et al.* 1993, 1999). Maintenance of ATP level during motility is initiated and provided by the hydrolysis of ATP which can be provided by the creatine kinase identified in our study. As seminal plasma and spermatozoa of rainbow trout exhibit enzyme activity to catabolize amino acids, they might serve as additional energy resources, especially for sperm motility activation (Lahnsteiner *et al.* 2009). Summarizing, the involvement of different enzymes associated with several energy production pathways and ATP production in spermatozoa likely agrees with the high and quick energy requirements for the initiation of fish sperm motility. Similar to changes in structural proteins, disturbances to metabolic enzymes could be a consequence of osmotic stress and be part of the mechanism resulting in lower quality of movement in hypoosmotic conditions.

Besides several proteins involved in sperm structure and metabolism, we have detected proteins engaged in other functions, including Ca^{2+} binding, signal transduction, protein folding and turnover. Since Ca^{2+} ions are necessary for triggering the motility of rainbow trout (Cosson *et al.* 1989), Ca^{2+} -binding proteins could be responsible for the mobilization of calcium ions in sperm. Disturbance of proteins involved in antioxidant protection might impair the scavenging or detoxifying of excess ROS in rainbow trout sperm during activation in hypoosmotic conditions indicated in this study, which leads to lower sperm motility parameters. In turn, signal transduction proteins could participate in initiation of sperm motility mediated by cAMP signaling pathways and serine/threonine-protein phosphatase 2A, which is known to regulate dynein phosphorylation in salmonids (Inaba 2002). Detailed roles of the proteins indicated need to be studied in further comprehensive studies.

Summing up, our results clearly demonstrate the different action of osmolality on rainbow trout spermatozoa motility. Activation in isosmotic conditions results in a higher quality of sperm movement in comparison to hypoosmotic conditions. During hypoosmotic activation of sperm motility with hatchery water, osmotic stress triggers oxidative stress and disturbances to structural proteins and metabolic enzymes. Sperm cells seem to be well protected in isosmotic conditions against osmotic and oxidative stress. Our results strongly suggest that comparative physiological and biochemical analysis of rainbow trout sperm characteristics in isosmotic and hypoosmotic conditions could be a useful model for studying the mechanism of sperm activation in salmonid fish.

Supplementary data

This is linked to the online version of the paper at <https://doi.org/10.1530/REP-19-0012>.

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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