Comparative Biochemistry and Physiology, Part A 241 (2020) 110634

Contents lists available at ScienceDirect

Comparative Biochemistry and Physiology, Part A

journal homepage: www.elsevier.com/locate/cbpa

The CatSper channel is present and plays a key role in sperm motility of the Atlantic salmon (*Salmo salar*)

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ARTICLE INFO

Keywords:
CatSper
Salmo salar
Motility
Sperm

ABSTRACT

Among all the Ca^{2+} channels, CatSper channels have been one of the most studied in sperm of different species due to their demonstrated role in the fertilization process. In fish sperm, the calcium channel plays a key role in sperm activation. However, the functionality of the CatSper channels has not been studied in any of the fish species. For the first time, we studied the relationship of the CatSper channel with sperm motility in a fish, using Atlantic salmon (*Salmo salar*) as the model. The results of our study showed that the CatSper channel in *Salmo salar* has chemical-physical characteristics similar to those reported for mammalian CatSper channels. In this work, it was shown that *Salmo salar* CatSper 3 protein has a molecular weight of approximately 55-kDa similar to *Homo sapiens* CatSper 3. *In silico* analyses suggest that this channel forms a heterotetramer sensitive to the specific inhibitor HC-056456, with a binding site in the center of the pore of the CatSper channel, hindering or preventing the influx of Ca^{2+} ions. The *in vitro* assay of the sperm motility inhibition of *Salmo salar* with the inhibitor HC-056456 showed that sperm treated with this inhibitor significantly reduced the total and progressive motility ($p < .0001$), demonstrating the importance of this ionic channel for this cell. The complementation of the *in silico* and *in vitro* analyses of the present work demonstrates that the CatSper channel plays a key role in the regulation of sperm motility in Atlantic salmon.

1. Introduction

Fish constitute the largest and most diverse group of vertebrates, with a wide diversity and reproduction strategies (Gallego and Asturiano, 2018). However, most of them share a common mode of fertilization, external fertilization, in which both female and male gametes are released into the environment (Crowe and Russell, 2009; Gallego and Asturiano, 2018). In both freshwater and saltwater fish, sperm are immobile in the seminal duct, and their motility is triggered when they come into contact with the aquatic environment where they experience a change in their physiology (Alavi et al., 2019). The reported variables that trigger sperm activation in fish include osmotic pressure, pH, temperature and ion concentrations such as K^+, Na^+, and Ca^{2+}(Alavi et al., 2019; Alavi and Cosson, 2006; Dzyuba and Cosson, 2014), where Ca^{2+} as the second messenger plays a key role in the initialization of this process in different fish species (Cosson, 2010). The influence of Ca^{2+} on sperm cells is conditioned by a wide variety of ion channels (Darszon et al., 1999), which are key elements in the fertilization process (Darszon et al., 2001). In the sperm of various species, several types of channels regulate the influx of Ca^{2+} cations such as voltage-gated calcium channels (VGCCs), transient receptor potential channel (TRP channels), cyclic nucleotide-gated ion channel (CNG channels) and cation channels of sperm (CatSper) (Darszon et al., 2011, 2006; Ishijima et al., 2002; Ren and Xia, 2010; Whitaker, 2006). Different studies on several species of fish such as rainbow trout (*Oncorhynchus mykiss*) (Kho et al., 2001; Tanimoto et al., 1994; TANIMOTO and MORISAWA, 1988), common carp (*Cyprinus carpio*) (Krasznai et al., 2003), Atlantic croaker (*Micropogonias undulatus*) (Detweiler and Thomas, 1998), and sterlet sturgeon (*Acipenser ruthenus*) (Bondarenko et al., 2017) have shown that calcium channels play a key role in regulating sperm motility.

CatSper channels are crucial in the regulation of sperm motility, and these have been shown to be essential during the fertilization process (Hildebrand et al., 2010; Ren and Xia, 2010; Sun et al., 2017), because

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https://doi.org/10.1016/j.cbpa.2019.110634
Received 21 October 2019; Received in revised form 30 November 2019; Accepted 6 December 2019
Available online 10 December 2019
1095-6433/

they constitute the main gateway of Ca^{2+} in the sperm of various species, thus playing a fundamental role in the control of flagellar movement (Kirichok et al., 2006; Lishko et al., 2011; Quill et al., 2001; Ren et al., 2001). These proteins are expressed only in sperm cells and are located in the main piece (Qi et al., 2007a). Some studies have revealed that CatSper are formed by four CatSper 1–4 subunits (Jin et al., 2005; Lobley et al., 2003; Quill et al., 2001), which are required for their functionality (Qi et al., 2007a). The models proposed for CatSper in mammalian sperm suggest that these proteins are formed by six transmembrane segments (Bystroff, 2018; Qi et al., 2007b), which form a heterotetrametric structure (Bystroff, 2018). The genes encoding CatSper proteins are present in various metazoan genomes, including aquatic animals (Cai and Clapham, 2008); however, the expression and functionality of these genes in non-mammalian species has been poorly studied.

In aquatic environments, sperm are guided to the egg through chemotaxis, similar to sea urchin sperm (Alvarez et al., 2012; Böhmer et al., 2005; Darszon et al., 2008; Guerrero et al., 2010; Kaupp et al., 2008; Wood et al., 2005). In a study carried out with sperm from *Arbacia punctulata*, it was shown that CatSper mediates the influx of Ca^{2+}, regulating the chemotaxis of these cells (Schiffer et al., 2014). In another more recent work on sperm from *Strongylocentrotus purpuratus*, it was proposed that CatSper is the main Ca^{2+} channel regulator of sperm motility in this species (Espinal-Enríquez et al., 2017). Studies on CatSper channels in fish remain to date a controversial area in a certain way. According to a genomic study, it was suggested that the genes encoding these channels could be eliminated during evolution in some fish species (Cai and Clapham, 2008). However, in a study carried out by Yanagimachi et al., it was demonstrated by immunohistochemical techniques that CatSper is present in some fish species such as black flounder (*Pleuronectes obscurus*), barfin flounder (*Verasper moseri*), rainbow trout (*Oncorhynchus mykiss*), Pacific herring (*Clupea pallasii*) and medaka (*Oryzias latipes*). (Yanagimachi et al., 2017). Despite this report, the structural features and the functionality of the CatSper channels have yet to be studied in fish. In this work, we study the structure and functionality of this ionic channel in a fish for the first time, using the Atlantic salmon (*Salmo salar*) as the model.

2. Materials and methods

2.1. Selection of CatSper protein sequences

The native proteins sequences of the CatSper type 1, 2, 3 and 4 of *Homo sapiens* (type 1 ID: Q8NEC5, type 2 ID: Q96P56, type 3 ID: Q86XQ3, type 4 ID: Q7RTX7) and *Mus musculus* (type 1 ID: Q91ZR5, type 2 ID: A2ARP9, type 3 ID: Q80W99, type 4 ID: Q8BVN3) available in the UniProt database (Bateman, 2019) were selected for this study.

2.2. Sequence alignments and phylogenetic analysis

For this analysis, we searched for homologous CatSper sequences within the Atlantic salmon (*Salmo salar*) proteome. Each of the sequences mentioned above were subjected to protein-protein alignments against the Atlantic salmon proteome (*Salmo salar* [taxid: 8030]; www.ncbi.nlm.nih.gov) using the BLASTp tool (Altschul et al., 1990). Only alignments with an identity percentage > 30% and an E-value < 10 were taken for homologous sequence selection. Subsequently, a phylogenetic analysis was performed with all sequences, using the Phylogeny.fr tool in its "One Click" mode (Dereeper et al., 2008). This mode involves the following steps: (1) Sequence alignment with MUSCLE version 3.8.31, (2) Curation with Gblocks version 0.91b (settings: Minimum length of a block after gap cleaning = 10; No gap positions were allowed in the final alignment; All segments with contiguous nonconserved positions bigger than 8 were rejected; Minimum number of sequences for a flank position = 85%), (3) Phylogenetic tree using the maximum likelihood method with PhyML version 3.1/3.0 aLRT (settings: Model = WAG; Statistical test = alrt; Number of categories = 4; Gamma = estimated; Invariable sites = estimated; Remove gaps = enabled) and (4) Tree Rendering with TreeDyn version 198.3 (settings: Conformation = rectangular; Legend = displayed; Branch annotation = bootstrap; Font = Times 8 normal).

2.3. Structure prediction and quality assessment

To date no native three-dimensional CatSper structures have been reported for any organism. Consequently, we modeled the CatSper tertiary (3D) structures of all the organisms included in this study. The secondary (2D) structure of all CatSper types was also predicted only for Atlantic salmon (*Salmo salar*). For this, the CCTOP (Constrained Consensus Topology Prediction server) program was used, which integrates the results from other methods for predicting 2D structures (Dobson et al., 2015). To select the most likely 2D structure in each case, the relative frequency was applied: Rf = n/N, where n is the number of methods that coincide in the prediction of transmembrane segments and N is the total of methods.

Homology modeling methods are based on the fact that evolutionary related proteins share a similar structure (Vitkup et al., 2001). If structural homologues do not exist, or exist but cannot be identified, models have to be constructed from scratch, this procedure is known as *ab initio* modeling. *Ab initio* are based on the global minimum free energy among all available protein conformations (Khor et al., 2015). For the prediction of all the CatSper 3D structures mentioned above, the Phyre2 program (Protein Homology/Analogy Recognition Engine) was used (Kelley et al., 2015). The intensive modeling mode was selected for the generation of all models. This modality combines homology modeling with *ab initio* techniques. Then, the models predicted with this program were refined with the GalaxyRefine tool for quality improvement (Heo et al., 2013). Subsequently, the quality of all the models were evaluated with the Rachamandram plot analysis, using the MolProbity tool (Chen et al., 2010) available on the SWISS-MODEL platform (Waterhouse et al., 2018).

2.4. Structural alignments

The structural alignments were carried out with the RaptorX Structure Alignment Server (Peng and Xu, 2011). The TM-score measures (Xu and Zhang, 2010) and root-mean-square deviation (RMSD) (Kufareva and Abagyan, 2012) were used to evaluate these alignments. A TM-score > 0.6 means that the aligned proteins share the same folding. By contrast, a TM-score < 0.4 means that they do not present the same folding and therefore the probability that they have the same function is low. On the other hand, two aligned proteins that have a low RMSD means that they have the same folding.

2.5. Molecular docking

In order to evaluate the affinity of the HC-056456 inhibitor of CatSper channels (Carlson et al., 2009) with the CatSper channel of Atlantic salmon sperm, a protein-ligand molecular docking was performed with the Autodock Vina program (Trott and Olson, 2010), which is integrated into PyRx software (Dallakyan and Olson, 2015). Since it has been reported that CatSper forms a heterotetramer consisting of Castper 4-1 and CatSper 2-3 dimers (Bystroff, 2018), in this study a unique sequence was constructed consisting of all CatSper types of Atlantic salmon in the following order: CatSper 4-1-2-3, which was used for the prediction of the 3D structure using the Phyre2 program (Kelley et al., 2015). To perform the molecular docking, an interaction region was established around the entire heterotetramer generated (Blind docking), setting the following parameters: center_x = 195.886281522; center_y = −28.3003325805; center_z = 92.042396671; size _x = 116.213091066; size _y = 112.164138989; size _z = 85.8060392462; and

exhaustiveness = 8.0. The 3D structure of the HC-056456 inhibitor (ChemSpider ID 498850) was downloaded from the ChemSpider database (Pence and Williams, 2010) and used as a ligand, after being optimized with the mmff94 force field (Halgren, 1996) available in PyRx software (Dallakyan and Olson, 2015). The heterotetramer-ligand interaction region was visualized with the use of PyMOL software (The PyMOL Molecular Graphics System, Version 2.3.1 Schrödinger, LLC).

2.6. Sperm sample collection and quality control

Sperm samples were extracted from four adult males (n = 4) of Atlantic salmon (*Salmo salar*) donated by the Hendrix Genetics Aquaculture SA fish farm located on Camino Rinconada km 6 – Sector Catripulli, Curarrehue, Chile (39°23′17″ S, 671°40′40″ W). The 3-year-old breeders (weight 7.1 ± 0.2 kg; length 82 ± 0.3 cm) were kept in 3000-L fibreglass tanks with recycled fresh water (500 L/h) at 8 °C and a natural photoperiod. The sperm was collected with a syringe by abdominal massage, always avoiding contamination with blood, urine, feces or water. Then the sperm was stored at 4 °C with oxygenation and absence of light in graduated and sterile plastic containers until use. For motility assessment, semen samples were previously diluted 1:100 in Storfish® commercial medium (pH 8.0) (IMV technologies) and 0.5 μL of cell suspension were activated on a glass slide in 6 μL of Powermilt® activator commercial solution (Universidad Católica de Temuco, Chile) supplemented with 0,25% (*w/v*) of Pluronic® (Sigma Aldrich) at pH 8.0 and immediately covered with a coverslip with a separation of approximately 0.04 mm (40 μm) from the slide to allow free swimming of spermatozoa. An analysis was performed immediately in a Computer-Assisted Sperm Analysis system (CASA) composed by a Motic BA310 negative phase contrast microscope at 100× magnification, Motic® camera and Integrated Sperm Analysis System software (AndroVision® system, Minitube®). The objects area (for detection of heads) was set from 3 to 30 μm^2, and the minimum speed threshold for motile cells was set at 15 μm/s. This analysis was carried out by making 10 captures for each sample and taking total and progressive motility as evaluation criteria. Motility percentage (> 80%) and cell concentration ($> 10 \times 10^6$ sperm/ml) were taken as inclusion criteria in this study. Total motility is the percentage of sperm referring to any type of movement. It also includes the non-progressive movement. Progressive motility refers to sperm that are swimming in a mostly straight line or in very large circles (Elia et al., 2010).

2.7. Sperm motility inhibition assay

Previously the semen was adjusted to a concentration of 10^6 sperm/ml with Storfish® commercial medium (diluted 1:100) (IMV technologies) at pH 8.0. Later, semen samples were treated with two concentrations of 15 μM and 20 μM of the HC-056456 inhibitor (Cat. No.: HY-112729, MEDCHEMEXPRESS) (Carlson et al., 2009), and subsequently incubated for 1 h at 4 °C, as well as the untreated samples (Control). Subsequently, sperm motility was evaluated using CASA, making 10 captures for each sample and taking the percentage of total and progressive motility as measures according the methodology described above.

2.8. Detection of CatSper 3 in sperm by Western blot

The presence of CatSper 3 proteins in *Salmo salar* was evaluated by Western blot. Previously, two cell washes were performed, where 200 μL of semen were resuspended in 600 μL to 1× of Storfish® commercial medium (pH 8.1; 261 mOsm; IMV, France), then the samples were centrifuged at 500 ×*g* for 5 min at 4 °C, always discarding the supernatant. Total protein extraction was performed using the protocol reported by Pini et al. (Pini et al., 2018) with slight modifications. Briefly, the pellets were resuspended in 600 μL of lysis buffer (62.5 mM tris, 2% (w/v), sodium dodecyl sulfate (SDS) and 1 mM PMSF) and then stirred at 15 Hz for one hour at room temperature. Subsequently, the lysates were centrifuged (7500 ×*g*; 15 min; room temperature) and the supernatant (protein extract) was stored at -80 °C until use.

Protein concentration was determined by the BCA assay (PierceTM BCA Protein Assay Kit, USA). Protein extracts (30 μg) were fractionated in a polyacrylamide gel electrophoresis with sodium dodecyl sulfate (SDS-PAGE) according to the method described by Laemmli (Laemmli, 1970), with a separating and concentrating gel of 12% and 4%, respectively. The Western blot assay was performed by transferring the proteins to a nitrocellulose membrane. The polyclonal anti-CatSper 3 antibody (PA5-50374, Invitrogen®) diluted 1:500 was used as the primary antibody, and the polyclonal Anti-rabbit IgG/HRP-linked Antibody (# 7074, Cell Signaling Technology®) diluted 1:2000 as the secondary. Western blot development was performed with TMB Stabilized Substrate for Horseradish Peroxidase (Cat. # W4121, Promega®). As a positive control a protein extract (total protein) of *Homo sapiens* sperm was used.

2.9. Statistic analysis

To assess the effect of the HC-056456 inhibitor on sperm motility, a variance analysis (ANOVA) and a Tukey test as an *a posteriori* test were performed, with a significance criterion of $p < .05$. The statistical analysis was performed with the use of the GraphPad PRISM 7.0 software.

3. Results

3.1. In silico analysis

The alignments made with BLASTp against the Atlantic salmon proteome database showed similarity percentages and E-values ranged from 37 to 45% and 7e-132 to 1e-89, respectively. This analysis made it possible to identify four homologous *Salmo salar* sequences for the different types of CatSper 1-4, with reference codes: XP_013984536.1, XP_013980195.1, XP_014053736.1 and XP_014033939.1, respectively. The phylogenetic analysis showed that regardless of the species, the native CatSper sequences referring to *Homo sapiens* and *Mus musculus* as their homologues in *Salmo salar* were grouped according to their type in different branches in the generated phylogenetic tree (Fig. 1).

The 2D structure analysis showed that the probability that CatSper homologues of Atlantic salmon are formed by six transmembrane segments is high given the relative frequencies obtained for each model, which were: 0.9, 0.4, 0.7 and 0.6 for XP_013984536.1, XP_013980195.1, XP_014053736.1 and XP_014033939.1, respectively.

All Catsper types, modeled and refined, showed a good quality according to the Ramachandran plot, with percentages in favored regions of: 93.66% (CatSper1 [*Homo sapiens*]); 91.56% (CatSper2 [*Homo sapiens*]); 93.65% (CatSper3 [*Homo sapiens*]); 95.85% (CatSper4 [*Homo sapiens*]); 94.37% (CatSper1 [*Mus musculus*]); 93.8% (CatSper2 [*Mus musculus*]); 94.41% (CatSper3 [*Mus musculus*]); 93.81% (CatSper4 [*Mus musculus*]); 91.38% (CatSper1 [*Salmo salar*]); 90.93% (CatSper2 [*Salmo salar*]); 92.27% (CatSper3 [*Salmo salar*]); and 95.0% (CatSper4 [*Salmo salar*]). The measurements calculated during the structural alignments carried out with the RaptorX Structure Alignment tool suggest the same functional relationship among all generated 3D models, RMSD = 1.85, TM-score = 0.85 (CatSper1), RMSD = 2.08; TM-score = 0.910 (CatSper2); RMSD = 1.33; TM-score = 0.954 (CatSper3); RMSD = 1.04; and TM-score = 0.948 (CatSper4) (Fig. 2).

On the other hand, the CatSper modeled from Atlantic salmon showed a typical structure of a Ca^{2+} channel formed by a central pore with negative charge (Fig. 3C). The molecular docking indicated that the HC-056456 inhibitor has a binding site inside the pore of the heterotetramer, with an affinity of −7.2 kcal/mol (Fig. 3A and B).

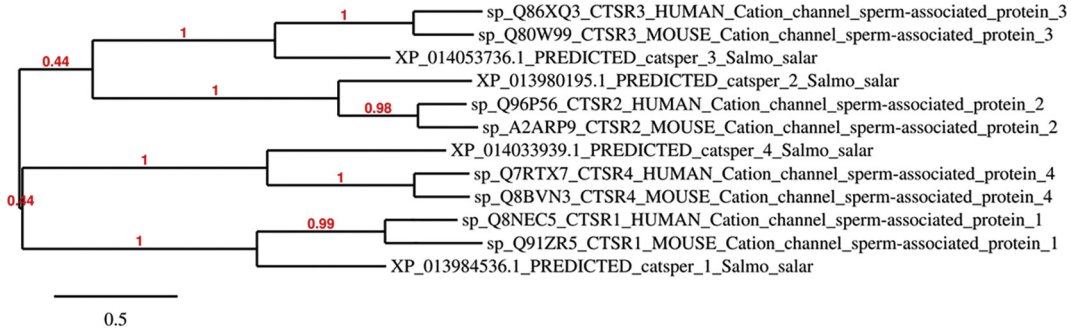

Fig. 1. Phylogenetic analysis of CatSper sequences corresponding to all organisms included in this study. All sequences were grouped according to the CatSper type in independent branches, suggesting a close phylogenetic relationship.

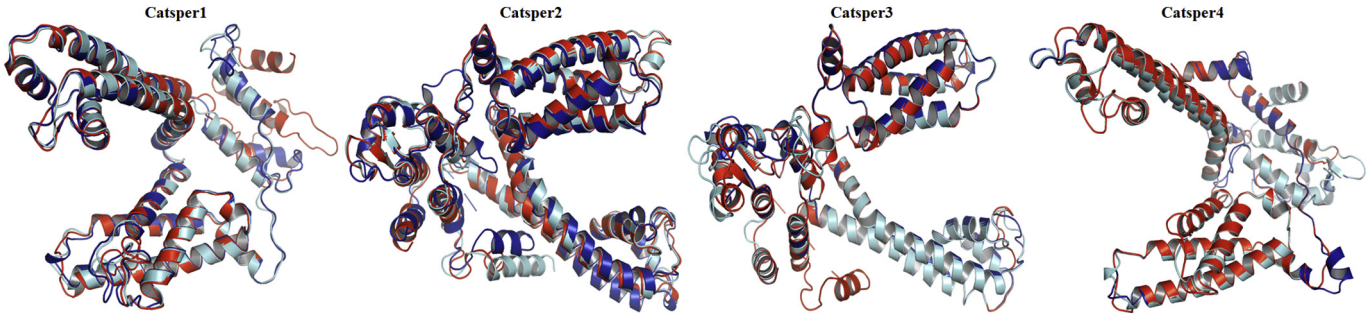

Fig. 2. The 3D alignment among all types of CatSper showed that the models superimpose, suggesting a common functional relationship (red: *Homo sapiens*, blue: *Mus musculus*, cyan: *Salmo salar*). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

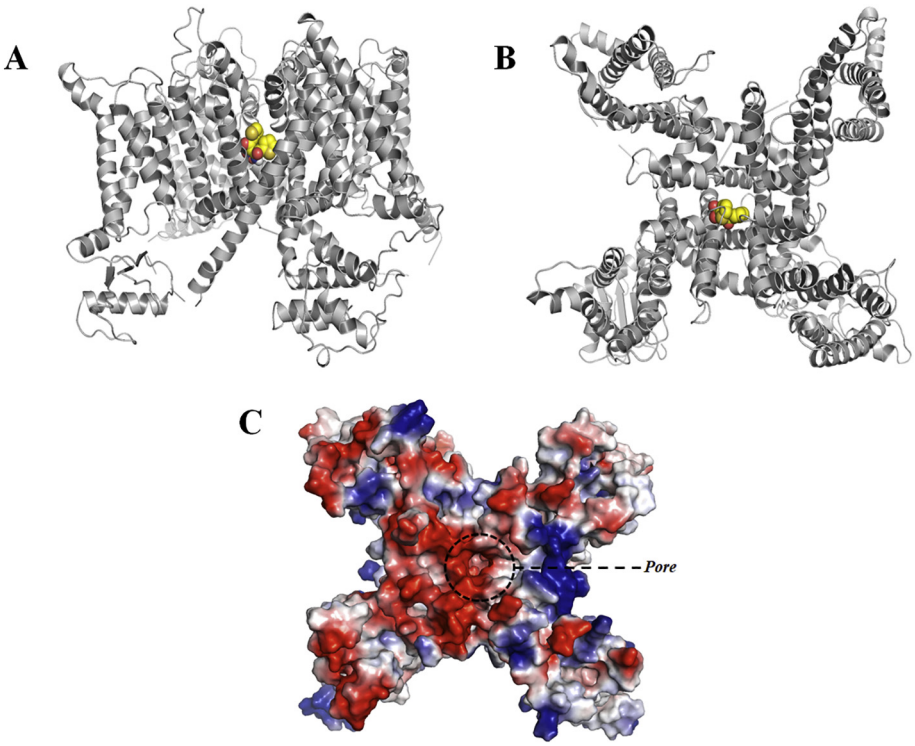

Fig. 3. Molecular docking between the CatSper heterotetramer of *Salmo salar* (in gray) and the inhibitor (in yellow). HC-056456 binds within the pore of the canal with a strong affinity (−7.2 kcal/mol). (A) Heterotetramer side view. (B) Heterotetramer top view. (C) Charge distribution on the heterotetramer (*red region:* negative charged, *blue region:* positive charged, *white region:* neutral). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

3.2. In vitro analysis

All sperm samples ($n = 4$) from Atlantic salmon evaluated by CASA showed motility percentages > 85% and cell concentrations > 10 × 10^6 sperm/ml. In the inhibition assay, a significant decrease in the total and progressive motilities was observed ($p < .0001$) compared to the control when the sperm were treated with both concentrations (15 μM and 20 μM) of the HC-056456 inhibitor (Fig. 4A). On the other hand, the Western blot result revealed the presence of a single protein band for each of the samples assessed, with molecular weights of approximately 55-kDa (Fig. 4A). In addition, when all sperm samples from *Salmo salar* ($n = 4$) were pooled and compared against a positive control (proteins extract from *Homo sapiens*), it was observed that both Catper 3 from *Homo sapiens* and *Salmo salar* have a similar molecular

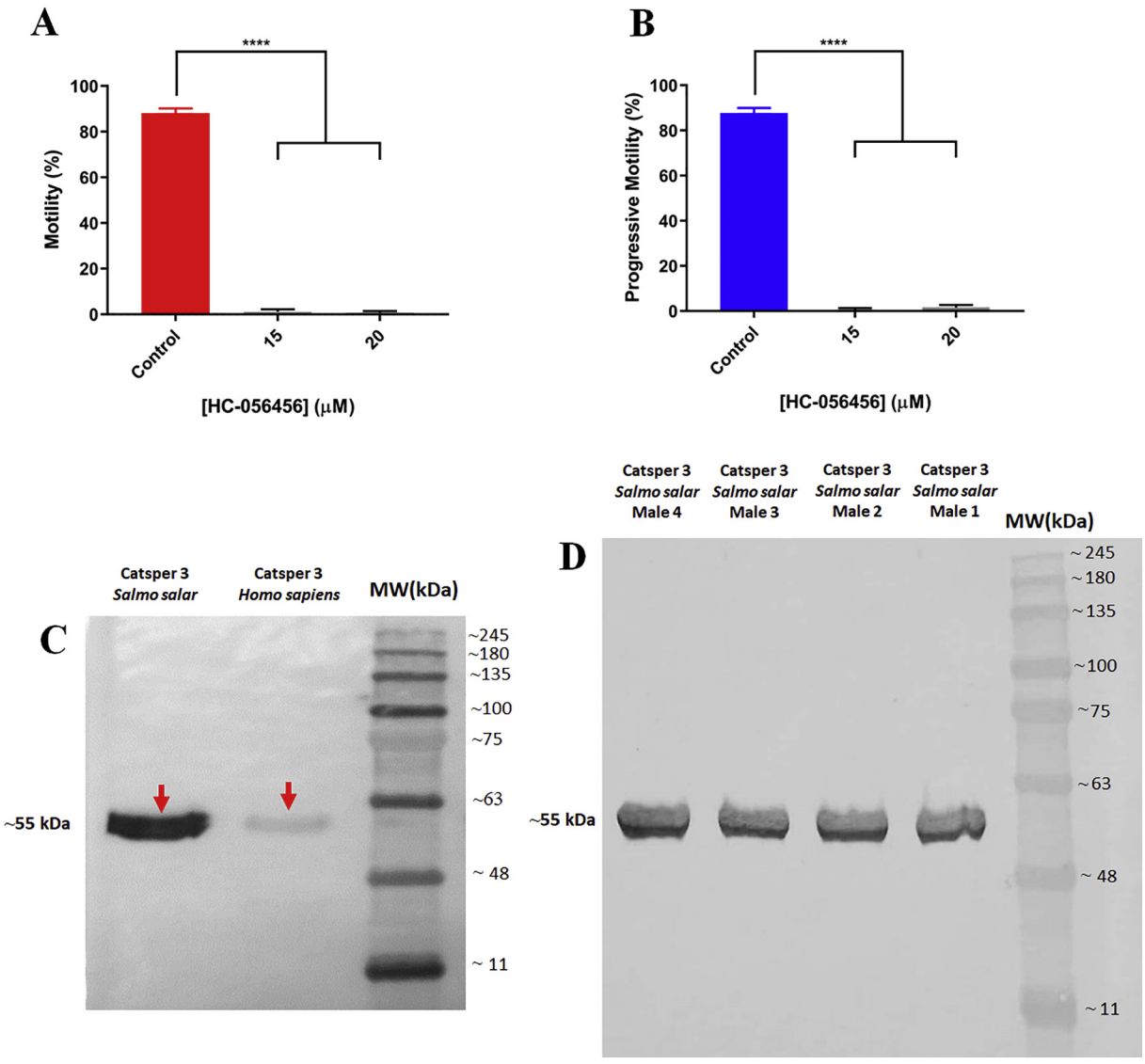

Fig. 4. Sperm samples from *Salmo salar* ($n = 4$) treated with different concentrations of the HC-056456 inhibitor significantly decrease their (A) total and (B) progressive motility (****$p < .0001$), respectively. Controls are sperm concentrations of 10^6 sperm/ml diluted 1:100 in Storfish® commercial medium at pH 8.0. (C) Western blot with each of the *Salmo salar* sperm protein extracts ($n = 4$) revealed the presence of CatSper 3 with a molecular weight of approximately 55-kDa. (D) CatSper 3 detection from a pulled protein extracts from *Salmo salar* sperm ($n = 4$), and a protein extract from *Homo sapiens* sperm as a positive control of the polyclonal anti-CatSper 3 antibody [PA5–50374, Invitrogen®] used in this study.

weight (Fig. 4B).

4. Discussion

CatSper is one of the most studied ion channels given its close relationship with sperm activation in different organisms (Lishko and Mannowetz, 2018), and its inactivity is strongly correlated with infertility (Hildebrand et al., 2010; Qi et al., 2007b; Saha et al., 2015; Sun et al., 2017). These channels are only found in the sperm (Quill et al., 2001) and located in the plasma membrane of the midpiece of flagellum, where the entry of Ca^{2+} ions are regulated (Sun et al., 2017). Although CatSper channels have been studied in various organisms, their study in fish remains a virtually unexplored area. According to an evolutionary genomic study conducted by Cai and Clapham in 2008, it was proposed that the genes encoding CatSper proteins in Agnatha (jawless fishes) and Teleostei (bony fishes) may not have provided any evolutionary advantage, and therefore were eliminated in these organisms (Cai and Clapham, 2008). However, recently Yanagimachi et al. demonstrated the presence of this channel through immunohistochemical techniques in some fish species such as black flounder (*Rhombosolea retiaria*), Pacific herring (*Clupea pallasii*), common medaka (*Oryzias latipes*) and rainbow trout (*Oncorhynchus mykiss*) by using a *Homo sapiens* polyclonal anti-CatSper3 antibody. In addition, the presence of a homologous sequence of CatSper 3 for Atlantic salmon (*Salmo salar*) based on genomic data was proposed (Yanagimachi et al., 2017).

In the present study, we investigated for the first time the relationship of the CatSper channel with motility in fish, using Atlantic salmon (*Salmo salar*) as the model, from the perspective of a robust *in silico* approach combined with *in vitro* analysis. In this work, it was evidenced that regardless of the evolutionary line that separates the Mammalia (Ex: *Homo sapiens* and *Mus musculus*) and Actinopterygii (Ex: *Salmo salar*) classes (Hurley et al., 2007), all CatSper proteins of Atlantic salmon were grouped into separate branches according to their type, which suggests a common functional relationship between these proteins (Fig. 1). The predicted 2D and 3D models suggest that Atlantic salmon CatSper proteins have six transmembrane segments and a heterotetramer arrangement (Fig. 3A, B and C), coinciding with the

proposed models for mammalian CatSper channels (Bystroff, 2018).

Structural alignments are one of the most used and robust *in silico* techniques to establish functional relationships between proteins based on their 3D structure (Aslam et al., 2016). The results of our structural alignments suggest a strong functional relationship among all the CatSper proteins evaluated due to the low RSMD and high TM-scores obtained. Given that the function of CatSper proteins as ion channels of *Homo sapiens* (Cooper and Phadnis, 2017) and *Mus musculus* (Vicens et al., 2014) are well known, according to this result the same function is encoded by CatSper proteins from *Salmo salar*.

In this study we investigated the inhibition of sperm motility in Atlantic salmon with the use of the HC-056456 inhibitor. It has been reported that this inhibitor causes asymmetry of the flagellar waveform, once it quickly and reversibly binds to the CatSper channel, resulting in the inhibition of motility (Carlson et al., 2009; Chávez et al., 2018; Mata-Martínez et al., 2018; Orta et al., 2018). Prior to the *in vitro* assay with this inhibitor, a molecular docking was performed between the modeled heterotetramer and the HC-056456 inhibitor, resulting in an affinity of −7.2 kcal/mol. Molecular docking studies with different voltage-gated calcium channels blockers report affinities between a range of −6.2 and −7.6 kcal/mol (Rahman et al., 2014). Given this range of reported values, the affinity value of our study (−7.2 kcal/mol), and the position of HC-056456 in the pore of the canal (Fig. 3A and B), it is expected that HC- 056456 provoke an antagonistic effect on the CatSper channel of Atlantic salmon acting as a blocker of this channel and hindering the influence of Ca^{2+} cations, thereby inhibiting sperm motility.

In the *in vitro* analysis of inhibition of sperm motility with HC-056456, it was observed that the concentrations reported by Carlson et al. in their study of CatSper inhibition in mice (Carlson et al., 2009) have a significant effect on inhibition of total and progressive sperm motility in Atlantic salmon ($p < .0001$) (Fig. 4C and D), which demonstrates the existence of this channel in this organism and confirms our *in silico* results. Finally, the detection analysis of CatSper 3 in Atlantic salmon by Western blot revealed the presence of a single protein band corresponding to a molecular weight of approximately 55-kDa, similar to that detected in the positive control of *Homo sapiens* (Fig. 4A and B), which reinforces our above-mentioned *in silico* and *in vitro* results regarding the presence of CatSper in *Salmo salar*. In the study carried out by Yanagimachi et al., also using a polyclonal anti-CatSper 3 antibody, a single band for rainbow trout (*Oncorhynchus mykiss*) and Pacific herring (*Clupea pallasii*) was revealed by Western blot assay. However, the relationship of this protein with sperm motility was not investigated (Yanagimachi et al., 2017). Additionally, we performed an alignment (BLASTp) of the sequence used as an immunogen to produce the polyclonal anti-CatSper 3 antibody (PA5-50374, Invitrogen®) against the *Salmo salar* database [taxid: 8030], and we found that this immunogen only aligns with three sequences corresponding to three isoforms of CatSper 3, which indicates the specificity of this antibody to detect CatSper3 in Atlantic salmon (See supplementary material).

To date, there was no report on the relationship of CatSper proteins with sperm motility in fish. In this investigation, we demonstrate for the first time that this ionic channel is present in Atlantic salmon and plays a key role in sperm motility. In future research, it would be interesting to address this issue for the study of sperm from other fish species.

5. Conclusions

Ionic calcium channels play an important role in sperm motility in fish. The present study constitutes the first report of the relationship of a CatSper channel with sperm motility in fish. We demonstrated for the first time, through a combined *in silico* and *in vitro* approach, the presence of the CatSper channel in *Salmo salar* spermatozoa as well as and its key function in the regulation of sperm motility.

Declaration of Competing Interest

The authors declare that there are no conflicts of interest.

Acknowledgments

This work was supported by the following projects: FONDECYT (No. 1180387), DI12-PEO1 DIUFRO, DIE14-0001 DIUFRO, FONDECYT/POSTDOCTORAL (No. 3180765), and UFRO Scholarship.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cbpa.2019.110634.

References

Alavi, S.M.H., Cosson, J., 2006. Sperm motility in fishes. (II) Effects of ions and osmolality: a review. Cell Biol. Int. https://doi.org/10.1016/j.cellbi.2005.06.004.
Alavi, S.M.H., Cosson, J., Bondarenko, O., Linhart, O., 2019. Sperm motility in fishes: (III) diversity of regulatory signals from membrane to the axoneme. Theriogenology. https://doi.org/10.1016/j.theriogenology.2019.06.038.
Altschul, S.F., Gish, W., Miller, W., Myers, E.W., Lipman, D.J., 1990. Basic local alignment search tool. J. Mol. Biol. https://doi.org/10.1016/S0022-2836(05)80360-2.
Alvarez, L., Dai, L., Friedrich, B.M., Kashikar, N.D., Gregor, I., Pascal, R., Kaupp, U.B., 2012. The rate of change in Ca 2+ concentration controls sperm chemotaxis. J. Cell Biol. https://doi.org/10.1083/jcb.201106096.
Aslam, N., Nadeem, A., Babar, M.E., Pervez, M.T., Aslam, M., Naveed, N., Hussain, T., Shehzad, W., Wasim, M., Bao, Z., Javed, M., 2016. The accuracy of protein structure alignment servers. Electron. J. Biotechnol. https://doi.org/10.1016/j.ejbt.2016.01.005.
Bateman, A., 2019. UniProt: a worldwide hub of protein knowledge. Nucleic Acids Res. https://doi.org/10.1093/nar/gky1049.
Böhmer, M., Van, Q., Weyand, I., Hagen, V., Beyermann, M., Matsumoto, M., Hoshi, M., Hildebrand, E., Kaupp, U.B., 2005. Ca2+ spikes in the flagellum control chemotactic behavior of sperm. EMBO J. https://doi.org/10.1038/sj.emboj.7600744.
Bondarenko, O., Dzyuba, B., Rodina, M., Cosson, J., 2017. Role of Ca2+ in the IVM of spermatozoa from the sterlet *Acipenser ruthenus*. Reprod. Fertil. Dev. https://doi.org/10.1071/RD16145.
Bystroff, C., 2018. Intramembranal disulfide cross-linking elucidates the super-quaternary structure of mammalian CatSpers. Reprod. Biol. https://doi.org/10.1016/j.repbio.2018.01.005.
Cai, X., Clapham, D.E., 2008. Evolutionary genomics reveals lineage-specific gene loss and rapid evolution of a sperm-specific ion channel complex: CatSpers and CatSperβ. PLoS One. https://doi.org/10.1371/journal.pone.0003569.
Carlson, A.E., Burnett, L.A., del Camino, D., Quill, T.A., Hille, B., Chong, J.A., Moran, M.M., Babcock, D.F., 2009. Pharmacological targeting of native CatSper channels reveals a required role in maintenance of sperm hyperactivation. PLoS One. https://doi.org/10.1371/journal.pone.0006844.
Chávez, J.C., De la Vega-Beltrán, J.L., José, O., Torres, P., Nishigaki, T., Treviño, C.L., Darszon, A., 2018. Acrosomal alkalization triggers ca 2+ release and acrosome reaction in mammalian spermatozoa. J. Cell. Physiol. https://doi.org/10.1002/jcp.26262.
Chen, V.B., Arendall, W.B., Headd, J.J., Keedy, D.A., Immormino, R.M., Kapral, G.J., Murray, L.W., Richardson, J.S., Richardson, D.C., 2010. MolProbity : all-atom structure validation for macromolecular crystallography. Acta Crystallogr. Sect. D Biol. Crystallogr. https://doi.org/10.1107/s0907444909042073.
Cooper, J.C., Phadnis, N., 2017. Parallel evolution of sperm hyper-activation Ca2+ channels. Genome Biol. Evol. https://doi.org/10.1093/gbe/evx131.
Cosson, J., 2010. Frenetic activation of fish spermatozoa flagella entails short-term motility, portending their precocious decadence. J. Fish Biol. https://doi.org/10.1111/j.1095-8649.2009.02504.x.
Crowe, T.P., Russell, R., 2009. Functional and Taxonomic Perspectives of Marine Biodiversity. https://doi.org/10.1007/b76710_27.
Dallakyan, S., Olson, A.J., 2015. Small-molecule library screening by docking with PyRx. Methods Mol. Biol. https://doi.org/10.1007/978-1-4939-2269-7_19.
Darszon, A., Labarca, P., Nishigaki, T., Espinosa, F., 1999. Ion channels in sperm physiology. Physiol. Rev. https://doi.org/10.1152/physrev.1999.79.2.481.
Darszon, A., Beltrán, C., Felix, R., Nishigaki, T., Treviño, C.L., 2001. Ion transport in sperm signaling. Dev. Biol. https://doi.org/10.1006/dbio.2001.0387.
Darszon, A., Acevedo, J.J., Galindo, B.E., Hernández-González, E.O., Nishigaki, T., Treviño, C.L., Wood, C., Beltrán, C., 2006. Sperm channel diversity and functional multiplicity. Reproduction. https://doi.org/10.1530/rep.1.00612.
Darszon, A., Guerrero, A., Galindo, B.E., Nishigaki, T., Wood, C.D., 2008. Sperm-activating peptides in the regulation of ion fluxes, signal transduction and motility. Int. J. Dev. Biol. https://doi.org/10.1387/ijdb.072550ad.
Darszon, A., Nishigaki, T., Beltran, C., Treviño, C.L., 2011. Calcium channels in the development, maturation, and function of spermatozoa. Physiol. Rev. https://doi.org/10.1152/physrev.00028.2010.
Dereeper, A., Guignon, V., Blanc, G., Audic, S., Buffet, S., Chevenet, F., Dufayard, J.-F.,

Guindon, S., Lefort, V., Lescot, M., Claverie, J.-M., Gascuel, O., 2008. Phylogeny.fr: robust phylogenetic analysis for the non-specialist. Nucleic Acids Res. https://doi.org/10.1093/nar/gkn180.

Detweiler, C., Thomas, P., 1998. Role of ions and ion channels in the regulation of Atlantic croaker sperm motility. J. Exp. Zool. https://doi.org/10.1002/(SICI)1097-010X(19980601)281:2<139::AID-JEZ8>3.0.CO;2-P.

Dobson, L., Reményi, I., Tusnády, G.E., 2015. CCTOP: a consensus constrained TOPology prediction web server. Nucleic Acids Res. https://doi.org/10.1093/nar/gkv451.

Dzyuba, V., Cosson, J., 2014. Motility of fish spermatozoa: from external signaling to flagella response. Reprod. Biol. https://doi.org/10.1016/j.repbio.2013.12.005.

Elia, J., Imbrogno, N., Delfino, M., Mazzilli, R., Rossi, T., Mazzilli, F., 2010. The importance of the sperm motility classes - future directions. Open Androl. J. 2, 42–43.

Espinal-Enríquez, J., Priego-Espinosa, D.A., Darszon, A., Beltrán, C., Martínez-Mekler, G., 2017. Network model predicts that CatSper is the main Ca2+ channel in the regulation of sea urchin sperm motility. Sci. Rep. https://doi.org/10.1038/s41598-017-03857-9.

Gallego, V., Asturiano, J.F., 2018. Fish sperm motility assessment as a tool for aquaculture research: a historical approach. Rev. Aquac. https://doi.org/10.1111/raq.12253.

Guerrero, A., Nishigaki, T., Carneiro, J., Tatsu, Yoshiro, Wood, C.D., Darszon, A., 2010. Tuning sperm chemotaxis by calcium burst timing. Dev. Biol. https://doi.org/10.1016/j.ydbio.2010.04.013.

Halgren, T.A., 1996. Merck molecular force field. V. Extension of MMFF94 using experimental data, additional computational data, and empirical rules. J. Comput. Chem. https://doi.org/10.1002/(SICI)1096-987X(199604)17:5/6<616::AID-JCC5>3.0.CO;2-X.

Heo, L., Park, H., Seok, C., 2013. GalaxyRefine: protein structure refinement driven by side-chain repacking. Nucleic Acids Res. https://doi.org/10.1093/nar/gkt458.

Hildebrand, M.S., Avenarius, M.R., Fellous, M., Zhang, Y., Meyer, N.C., Auer, J., Serres, C., Kahrizi, K., Najmabadi, H., Beckmann, J.S., Smith, R.J.H., 2010. Genetic male infertility and mutation of CATSPER ion channels. Eur. J. Hum. Genet. https://doi.org/10.1038/ejhg.2010.108.

Hurley, I.A., Mueller, R.L., Dunn, K.A., Schmidt, E.J., Friedman, M., Ho, R.K., Prince, V.E., Yang, Z., Thomas, M.G., Coates, M.I., 2007. A new time-scale for ray-finned fish evolution. Proc. R. Soc. B Biol. Sci. https://doi.org/10.1098/rspb.2006.3749.

Ishijima, S., Baba, S.A., Mohri, H., Suarez, S.S., 2002. Quantitative analysis of flagellar movement in hyperactivated and acrosome-reacted golden hamster spermatozoa. Mol. Reprod. Dev. https://doi.org/10.1002/mrd.10017.

Jin, J.-L., O'Doherty, A.M., Wang, S., Zheng, H., Sanders, K.M., Yan, W., 2005. Catsper3 and Catsper4 encode two cation channel-like proteins exclusively expressed in the testis. Biol. Reprod. https://doi.org/10.1095/biolreprod.105.045468.

Kaupp, U.B., Kashikar, N.D., Weyand, I., 2008. Mechanisms of sperm chemotaxis. Annu. Rev. Physiol. https://doi.org/10.1146/annurev.physiol.70.113006.100654.

Kelley, L.A., Mezulis, S., Yates, C.M., Wass, M.N., Sternberg, M.J.E., 2015. The Phyre2 web portal for protein modeling, prediction and analysis. Nat. Protoc. https://doi.org/10.1038/nprot.2015.053.

Kho, K.H., Tanimoto, S., Inaba, K., Oka, Y., Morisawa, M., 2001. Transmembrane cell signaling for the initiation of trout sperm motility: roles of ion channels and membrane hyperpolarization for cyclic AMP synthesis. Zool. Sci. https://doi.org/10.2108/zsj.18.919.

Khor, B.Y., Tye, G.J., Lim, T.S., Choong, Y.S., 2015. General overview on structure prediction of twilight-zone proteins. Theor. Biol. Med. Model. https://doi.org/10.1186/s12976-015-0014-1.

Kirichok, Y., Navarro, B., Clapham, D.E., 2006. Whole-cell patch-clamp measurements of spermatozoa reveal an alkaline-activated Ca2+ channel. Nature. https://doi.org/10.1038/nature04417.

Krasznai, Z., Morisawa, M., Morisawa, S., Krasznai, Z.T., Trón, L., Gáspár, R., Márián, T., 2003. Role of ion channels and membrane potential in the initiation of carp sperm motility. Aquat. Living Resour. https://doi.org/10.1016/S0990-7440(03)00054-8.

Kufareva, I., Abagyan, R., 2012. Methods of protein structure comparison. Methods Mol. Biol. https://doi.org/10.1007/978-1-61779-588-6_10.

Laemmli, U.K., 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature. https://doi.org/10.1038/227680a0.

Lishko, P.V., Mannowetz, N., 2018. CatSper: a unique calcium channel of the sperm flagellum. Curr. Opin. Physiol. https://doi.org/10.1016/j.cophys.2018.02.004.

Lishko, P.V., Botchkina, I.L., Kirichok, Y., 2011. Progesterone activates the principal Ca 2+ channel of human sperm. Nature. https://doi.org/10.1038/nature09767.

Lobley, A., Pierron, V., Reynolds, L., Allen, L., Michalovich, D., 2003. Identification of human and mouse CatSper3 and CatSper 4 genes: characterisation of a common interaction domain and evidence for expression in testis. Reprod. Biol. Endocrinol. https://doi.org/10.1186/1477-7827-1-53.

Mata-Martínez, E., Darszon, A., Treviño, C.L., 2018. pH-dependent Ca+2 oscillations prevent untimely acrosome reaction in human sperm. Biochem. Biophys. Res. Commun. https://doi.org/10.1016/j.bbrc.2018.02.042.

Orta, G., De La Vega-Beltran, J.L., Martín-Hidalgo, X.D., Santi, C.M., Visconti, P.E., Darszon, X.A., 2018. CatSper channels are regulated by protein kinase A. J. Biol. Chem. https://doi.org/10.1074/jbc.RA117.001566.

Peng, J., Xu, J., 2011. Raptorx: exploiting structure information for protein alignment by statistical inference. Proteins Struct. Funct. Bioinforma. https://doi.org/10.1002/prot.23175.

Pini, T., Rickard, J.P., Leahy, T., Crossett, B., Druart, X., de Graaf, S.P., 2018. Cryopreservation and egg yolk medium alter the proteome of ram spermatozoa. J. Proteome. https://doi.org/10.1016/j.jprot.2018.04.001.

Qi, H., Moran, M.M., Navarro, B., Chong, J.A., Krapivinsky, G., Krapivinsky, L., Kirichok, Y., Ramsey, I.S., Quill, T.A., Clapham, D.E., 2007a. All four CatSper ion channel proteins are required for male fertility and sperm cell hyperactivated motility. Proc. Natl. Acad. Sci. https://doi.org/10.1073/pnas.0610286104.

Qi, Huayu, Moran, M.M., Navarro, B., Chong, J.A., Krapivinsky, G., Krapivinsky, L., Kirichok, Y., Ramsey, I.S., Quill, T.A., Clapham, D.E., 2007b. All four CatSper ion channel proteins are required for male fertility and sperm cell hyperactivated motility. Proc. Natl. Acad. Sci. U. S. A. https://doi.org/10.1073/pnas.0610286104.

Quill, T.A., Ren, D., Clapham, D.E., Garbers, D.L., 2001. A voltage-gated ion channel expressed specifically in spermatozoa. Proc. Natl. Acad. Sci. U. S. A. https://doi.org/10.1073/pnas.221454998.

Rahman, T., Cai, X., Brailoiu, G.C., Abood, M.E., Brailoiu, E., Patel, S., 2014. Two-pore channels provide insight into the evolution of voltage-gated Ca2+and Na+ channels. Sci. Signal. https://doi.org/10.1126/scisignal.2005450.

Ren, D., Xia, J., 2010. Calcium signaling through CatSper channels in mammalian fertilization. Physiology. https://doi.org/10.1152/physiol.00049.2009.

Ren, D., Navarro, B., Perez, G., Jackson, A.C., Hsu, S., Shi, Q., Tilly, J.L., Clapham, D.E., 2001. A sperm ion channel required for sperm motility and male fertility. Nature. https://doi.org/10.1038/35098027.

Saha, S., Talukdar, K., Chakraborty, A.K., 2015. The role of CatSper1 and CatSper2 ion channels in male fertility and infertility. Procedia Mater. Sci. https://doi.org/10.1016/j.mspro.2015.06.088.

Schiffer, C., Müller, A., Egeberg, D.L., Alvarez, L., Brenker, C., Rehfeld, A., Frederiksen, H., Wäschle, B., Kaupp, U.B., Balbach, M., Wachten, D., Skakkebaek, N.E., Almstrup, K., Strünker, T., 2014. Direct action of endocrine disrupting chemicals on human sperm. EMBO Rep. https://doi.org/10.15252/embr.201438869.

Sun, X. hong, Zhu, Y. ying, Wang, L., Liu, H. ling, Ling, Y., Li, Z. li, Sun, L. bo, 2017. The Catsper channel and its roles in male fertility: a systematic review. Reprod. Biol. Endocrinol. https://doi.org/10.1186/s12958-017-0281-2.

TANIMOTO, S., MORISAWA, M., 1988. Roles for potassium and calcium channels in the initiation of sperm motility in rainbow trout (K+/Ca2+/channel/sperm motility/rainbow trout). Develop. Growth Differ. https://doi.org/10.1111/j.1440-169X.1988.00117.x.

Tanimoto, S., Nakazawa, T., Kudo, Y., Morisawa, M., 1994. Implication that potassium flux and increase in intracellular calcium are necessary for the initiation of sperm motility in salmonid fishes. Mol. Reprod. Dev. https://doi.org/10.1002/mrd.1080390409.

Trott, O., Olson, A.J., 2010. Autodock vina. J. Comput. Chem. https://doi.org/10.1002/jcc.

Vicens, A., Tourmente, M., Roldan, E.R.S., 2014. Structural evolution of CatSper1 in rodents is influenced by sperm competition, with effects on sperm swimming velocity. BMC Evol. Biol. https://doi.org/10.1186/1471-2148-14-106.

Vitkup, D., Melamud, E., Moult, J., Sander, C., 2001. Completeness in structural genomics. Nat. Struct. Biol. https://doi.org/10.1038/88640.

Waterhouse, A., Bertoni, M., Bienert, S., Studer, G., Tauriello, G., Gumienny, R., Heer, F.T., De Beer, T.A.P., Rempfer, C., Bordoli, L., Lepore, R., Schwede, T., 2018. SWISS-MODEL: homology modelling of protein structures and complexes. Nucleic Acids Res. https://doi.org/10.1093/nar/gky427.

Whitaker, M., 2006. Calcium at fertilization and in early development. Physiol. Rev. https://doi.org/10.1152/physrev.00023.2005.

Wood, C.D., Nisihigaki, T., Furuta, T., Baba, S.A., Darszon, A., 2005. Real-time analysis of the role of Ca 2 in flagellar movement and motility in single sea urchin sperm. J. Cell Biol. https://doi.org/10.1083/jcb.200411001.

Xu, J., Zhang, Y., 2010. How significant is a protein structure similarity with TM-score = 0.5? Bioinformatics. https://doi.org/10.1093/bioinformatics/btq066.

Yanagimachi, R., Harumi, T., Matsubara, H., Yan, W., Yuan, S., Hirohashi, N., Iida, T., Yamaha, E., Arai, K., Matsubara, T., Andoh, T., Vines, C., Cherr, G.N., 2017. Chemical and physical guidance of fish spermatozoa into the egg through the micropyle. Biol. Reprod. https://doi.org/10.1093/biolre/iox015.