Genomic Identification, Expression Profiling and Functional Characterisation of CatSper Channels in the Bovine[1]

Running title

CatSper channels in bovine sperm

Summary sentence

Effect of Blocking Calcium Channels on Hyperactivation and Rheotactic Response

Key words

Sperm, calcium, rheotaxis, hyperactivation, fertility, comparative reproduction, bull

Gillian P. Johnson[2,4,5], Anne-Marie English[2,4], Sinead Cronin[2], David A. Hoey[4,5,6], Kieran G. Meade[3], Sean Fair[2]

[2]Laboratory of Animal Reproduction, Department of Biological Sciences, School of Natural Sciences, Faculty of Science and Engineering, University of Limerick, Limerick, V94 PH61, Ireland

[1] The authors gratefully acknowledge the financial support from the Irish Research Council (Dublin, Ireland; GOIPG/2014/1463), a European Research Council (ERC) Starting Grant (#336882) and a Science Foundation Ireland ERC Support Grant SFI 13/ERC/L2864 (to D.A.H.).
[2] Corresponding author: Dr. Sean Fair, Laboratory of Animal Reproduction, Department of Biological Sciences, School of Natural Sciences, Faculty of Science and Engineering, University of Limerick, Limerick, V94 PH61, Ireland Tel.: +353 61 202548; Fax: +353 61 331490; Email sean.fair@ul.ie

[3]Animal and Bioscience Research Department, Animal and Grassland Research and Innovation Centre, Teagasc, Grange, Dunsany, Meath, C15 PW93, Ireland

[4]Dept. of Mechanical and Manufacturing Engineering, School of Engineering, Trinity College Dublin, Dublin 2, D02 DK07, Ireland

[5]Trinity Centre for Bioengineering, Trinity Biomedical Sciences Institute, Trinity College, Dublin, D02 R590, Ireland

[6]Advanced Materials and Bioengineering Research Centre, Trinity College Dublin & RCSI, Dublin 2, D02 VN51, Ireland

Abstract

Cation channel of sperm (CatSper), are sperm-specific calcium channels with identified roles in the regulation of sperm function in humans, mice and horses. We sought to employ a comparative genomics approach to identify conserved *CATSPER* genes in the bovine genome, and profile their expression in reproductive tissue. We hypothesised that CATSPER proteins expressed in bull testicular tissue mediates sperm hyperactivation and their rheotactic response in the reproductive tract of the cow. Bioinformatic analysis identified all four known *CATSPER* genes (*CATSPER 1-4*) in the bovine genome and profiling by qPCR identified site-specific variation in mRNA expression for all 4 genes along the reproductive tract of the bull. Using a novel antibody against CATSPER 1, protein expression was confirmed and localized to the principal piece of bull sperm, in agreement with what has been reported in other species. Subsequent treatment of bull sperm with either the calcium chelator ethylene glycol tetraacetic acid (EGTA); mibefradil, a specific blocker of CatSper channels in human sperm; or CATSPER1 antibody all significantly inhibited caffeine induced hyperactivation and the rheotactic response, supporting the concept that the calcium influx occurs via CatSper channels. Taken together, the work here provides novel insights into expression and function of CatSper channels in bull testicular tissue and in the function of ejaculated sperm.

Introduction

Despite tens of millions to billions (species dependent) of sperm being deposited in the vagina/cervix after ejaculation, only a few hundred sperm are thought to be present in the ampulla of the oviducts at the time of fertilisation [1]. It is now apparent that sperm transport in the female reproductive tract is facilitated by a range of physiological mechanisms including rheotaxis, chemotaxis, thermotaxis as well as smooth muscle contractions of the female reproductive tract [2-4]. One of the least studied of these is rheotaxis, which is a cell's preference to swim with or against fluid flow, and has recently been reported as a mechanism for directing sperm towards the oocyte [3]. Positive rheotaxis is the tendency of sperm to swim against the flow and has been lightly observed over the past 50 years, but only recently has rheotaxis gained attention as a guiding mechanism [3, 5]. Rheotaxis plays a fundamental role in directing sperm towards the site of fertilisation, as sperm swim against the retrograde flow of mucus secreted under the influence of oestrogen in the lead up to ovulation [3, 6, 7]. In mice, it has been shown that positive rheotaxis occurs as a result of the spiral rotation of the sperm tail, leading to an increased amplitude of the tail waves and flagellar force, orientating the sperm upstream. This mechanism has been shown to be dependent on the influx of extracellular calcium (Ca^{2+}) [3]. Bull sperm have been shown to exhibit positive rheotaxis and can change their trajectory with respect to a change in fluid flow direction [7]. While bull sperm and sperm of other species have been shown to display positive rheotaxis, the mechanisms by which this is mediated are unclear.

Hyperactivation is characterised by a high amplitude, asymmetrical beating pattern (whip-like movement) of the sperm tail [8]. It is hypothesized that hyperactivation assists sperm in pulling away from the oviductal epithelium and increases swimming efficiency in viscous mucus [8, 9]. As with rheotaxis, the exact molecular mechanisms by which hyperactivation is mediated has yet to be elucidated, however, it has been demonstrated that Ca^{2+} and cyclic adenosine monophosphate (cAMP) are two key factors in the regulation of hyperactivation of mammalian sperm [10].

Intracellular increases in Ca^{2+} have been reported in hyperactivated flagella of hamster and bull sperm [11], where a high concentration of caffeine was used to increase intracellular Ca^{2+} [12].

In most cells, entry of external Ca^{2+} occurs through several types of Ca^{2+} channels: mainly voltage-activated channels and store-operated channels [13, 14] while, intracellular Ca^{2+} may be released from internal stores via receptor-operated channels. Inositol-1,4,5-trisphosphate (IP3) receptors (IP3R) and ryanodine receptors represent the two main intracellular Ca^{2+} channels responsible for releasing stored Ca^{2+}. In sperm of many mammals, members of a specific transmembrane Ca^{2+} channel family, cation channel of sperm (CatSper), also play an important role [15]. CatSper are weakly voltage-dependent, Ca^{2+} selective, pH-sensitive ion channels that control the entry of positively charged Ca^{2+} ions into sperm [16]. CatSper is composed of four separate pore-forming α (alpha) subunits; these are CatSper 1–4 and three additional auxiliary subunits: CatSper β (beta), CatSper γ (gamma) and CatSper δ (delta). The complexity of the channel due to the several subunits, seems to be necessary for its functional co-ordination, localization to the flagella, and sensitivity to intracellular pH, progesterone, prostaglandins, odorants, and to a potential range of other proteins and signalling molecules [16, 17]. CatSper channels have been shown to localize to the principal piece of the flagellum and are involved in the regulation of sperm function and male fertility in humans, mice and horses [8, 18, 19]. In mice, hyperactivated sperm motility is dependent upon the presence of CatSper channels, where sperm lacking in any one of the CatSper subunits fail to develop functional CatSper Ca^{2+} currents, and therefore, are unable to hyperactivate [8, 17, 20]. Mutations in human *CATSPER* genes are associated with infertility and abnormal sperm motility [21]. While CatSper channels have been identified and characterised in humans, stallions and mice [8, 18, 19], they have yet to be identified in the bull.

We hypothesised that CatSper channels are present in bull sperm and they play a role in hyperactivation and rheotactic response. Therefore, the aim of this study was to use a comparative genomics approach to identify and characterise the evolutionary orthologs of *CATSPER* genes in

67 the bovine genome and to investigate the effect of CatSper agonists and antagonists as well as
68 extracellular calcium on bull sperm hyperactivation and rheotaxis.

Materials and Methods

Bioinformatic Identification of Bovine *CATSPER* Orthologs

Orthology searches using the basic local alignment search tool BLAST [22] were performed for four known human and mouse *CATSPER* gene sequences (*CATSPER 1-4*) in the bovine genome (version: bosTau8). The bioinformatic tool, BLAST-Like Alignment Tool (BLAT; UCSC Genome Browser), was used to determine the chromosomal position of the four orthologous bovine genes identified. Phylogenetic analysis was also performed to investigate the evolutionary relationships between the four novel bovine *CATSPER* genes and their evolutionary orthologs using MEGA software [23, 24]. Bootstrap resampling was carried out 1000 times. Bovine *CATSPER* genes were annotated on the basis of sequence similarity and phylogenetic relationships to previously-described *CATSPER* sequences in humans and mice to maintain consistency in the comparative analysis of *CATSPERs* with other species. A multiple sequence alignment for all genes was performed using T-coffee [25] and annotated using Jalview [26].

Reproductive Tissue Collection

To characterise the expression profile of *CATSPER* genes, bull reproductive tract segments (tissues and sperm), including parenchyma testis, rete testis and the different segments of the epididymis (caput, corpus and cauda) were collected from sexually-mature beef bulls (n=4) within 20 min of slaughter. All segments ~~tissue samples~~ were immediately snap frozen in liquid nitrogen and transported to the laboratory for RNA extraction. The rationale for this experiment was simply to assess if *CATSPER* genes were expressed and, if so, which of the *CATSPER* genes were most highly expressed in the bull.

RNA Extraction and cDNA Synthesis

Total ribonucleic acid (RNA) was extracted from all tissues using a homogenizer to disrupt cells in buffer RLT, supplied with the RNeasy mini kit (Qiagen), according to the manufacturer's instructions. All samples were DNA-digested to remove genomic DNA using Qiagen's on-column DNase and eluted with water. RNA quantity was assessed using a Nanodrop spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA), whereas, the quality was determined with the use of an Agilent Bioanaylser (Agilent Technologies, Santa Clara, CA, USA). Complementary DNA (cDNA) was synthesised using an Applied Biosystems cDNA reverse-transcription kit (Life Technologies, Carlsbad, CA, USA) and an Eppendorf Mastercycler (Eppendorf, Hamburg, Germany).

Primer design, Quantitative Real-Time Polymerase Chain Reaction (qPCR)

Nucleotide sequences were retrieved from the University of California, Santa Cruz (UCSC) Genome Browser and Primer3 used for primer design (Table 1). Primers were designed to be intron spanning and were commercially synthesised (Sigma Aldrich, St Louis, MO, USA). Quantitative real-time polymerase chain reaction (qRT-PCR) was performed using a 20 µl reaction mix containing 10 µl SYBR green PCR MasterMix (Invitrogen Ltd, Paisley, UK), 2.5 µl primer and dH_2O mix, 5.5 µl dH2O and 2 µl sample. Plates were run in an ABI 7500 Fast Thermocycler (Life Technologies, Carlsbad, CA, USA). The cycle parameters were as follows: Uracil N-glycosylase (UNG) activation was run for 2 min at 50°C, DNA polymerase activation for 10 min at 95°C, the melt cycle was run for 15 s at 95°C and the annealing–extending cycle for 1 min at 60°C. A no-template control (NTC) was run in each 96-well plate to confirm the absence of gDNA contamination. Levels of expression of the gene of interest were compared with the average of the two reference genes, glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and beta actin (ACTb). Normalising gene expression to multiple reference genes in order to give a more reliable baseline

for the calculation of relative gene expression using qRT-PCR is common practice especially when small changes in gene expression are being reported [27].

In Situ Immunocytochemistry for the CATSPER1 Protein

Fresh bull semen from a commercial bull stud was collected using an artificial vagina and diluted to 2 x 10^6 sperm/ml in phosphate buffered saline (PBS; Sigma Aldrich, St Louis, MO, USA). Cells were fixed in 4% paraformaldehyde, and washed with PBS before being permeabilised with Triton X 100 (0.2%) for 15 min at room temperature (RT). After rinsing in PBS, cells were blocked using blocking buffer (0.4 g BSA + 2 µl Triton X 100 + 10 ml PBS) for 1 h at RT. Immunostaining was carried out using primary antibody targeted against CATSPER1 (1:100; ab203626, Abcam, Cambridge, United Kingdom) at 4°C overnight. The immunogen sequence of the CATSPER1 antibody used was MDSRAQGAWY. The homology between this and the bovine CATSPER1 sequence, was 90%. After three 5 min washes in PBS, sperm were incubated for 1 h with goat anti-mouse IgG Alexa Fluor 488 (1:500; Invitrogen, California, United States) as a secondary antibody. Nuclei were counterstained with DAPI. Cells were imaged with an Olympus IX83 (Norfolk, USA) inverted microscope equipped with a 40X objective.

Sperm Preparation

Frozen-thawed sperm from Holstein bulls (n=3) of proven fertility were used in all the functional experiments. Semen straws were thawed in a water bath at 39°C for 30 sec. For each functional assessment, one straw per bull, of which there where three, were thawed. The resulting volume to run the assessment consisted of a pool of three straws coming from three bulls to eliminate the inter-bull variability. All sperm were diluted in TALP media [28].

Effect of Extracellular Ca^{2+} on Hyperactivation and Rheotactic Response

The aim of this experiment was to investigate the role of extracellular calcium on sperm hyperactivation and rheotaxis. Preliminary experiments were carried out to assess the optimum concentration of all agonists/antagonists (data not shown). To investigate this, extracellular Ca^{2+} was ablated using ethylene glycol tetraacetic acid (EGTA) which chelates Ca^{2+} in the media [29]. Motility of the frozen-thawed sperm were analysed objectively by computer assisted sperm analysis (CASA;Sperm Class Analyzer, Microptic, Viladomat, Barcelona, Spain). A droplet of diluted sperm (10 µl) from each sample described above was placed on a pre-warmed slide, a pre-warmed cover slip was added and analysed for sperm motion and kinematic parameters using factory CASA (bull) settings. At least three randomly selected microscopic fields and a minimum of 100 sperm per treatment was assessed. Any samples with post thaw progressive motility less than 30% were not used in experiments. Thawed sperm were incubated with either (i) 2 mM EGTA (ii) 5 mM caffeine (phosphodiesterase inhibitor but also an agonist for extracellular Ca^{2+} influx [30] (iii) 2 mM EGTA in combination with 5 mM caffeine or (iv) no treatment, for 10 min before hyperactivation and rheotaxis were assessed. Hyperactivation was assessed using a phase-contrast microscope (CX41; Olympus, Hamburg, Germany) at a magnification of 40X. A droplet of diluted sperm (10 µL at a concentration of 25 x 10^6 sperm/mL) was placed on a pre-warmed slide, covered with a pre-warmed cover slip and assessed subjectively by counting 100 motile sperm for each treatment. Hyperactivation was expressed as the percentage of ~~live and~~ motile sperm which displayed hyperactivated motility. Hyperactivation was characterised by high amplitude, asymmetrical beating pattern of the sperm tail [31]. This manifested in a characteristic figure of eight swimming trajectory. To assess sperm rheotactic response, sperm were loaded into the starting well (50 µL at a concentration of 25 x 10^6 sperm/mL) of a specialised microfluidic device (micro-channel size of 300 µm wide, 100 µm deep and 30 mm in length) with a flow rate of 30 µm/sec. The number of sperm which swam passed the 10 mm mark in the micro-channel at 10 min were assessed. This

experiment was replicated three times. ~~In addition to rheotactic response and hyperactivation, motility was assessed to ensure treatment did not have an effect on overall motility.~~

Effect of Blocking Calcium Channels on Hyperactivation and Rheotactic Response

The aim of this experiment was to assess the role of calcium channels in the hyperactivation and the rheotactic response of bull sperm. To achieve this, a Ca^{2+} channel antagonist (Mibefradil) was utilised to block all sperm Ca^{2+} channels [8]. The concentration of mibefradil was chosen following a dose response effect of 1, 5 and 10 µM mibefradil on hyperactivation. Caffeine (5 mM) was used to induce hyperactivation as a positive control in all experiments. Sperm were incubated with (i) mibefradil (5 µM) (ii) caffeine (5 mM), (iii) mibefradil (5 µM) in combination with caffeine (5 mM) or (iv) no treatment, for 10 min prior to the assessment of rheotactic response and hyperactivation as described above. This experiment was replicated three times. Motility of the frozen-thawed sperm were analysed as described above.

Effect of CatSper Channels on Hyperactivation and Rheotactic Response

The aim of this experiment was to assess the effect of CatSper channels on hyperactivation and rheotactic response of bull sperm. To achieve this, CATSPER1 antibody (Ab) was used to specifically block CatSper1. An initial dose response hyperactivation test was carried out using; 0.8, 4 and 20 µg/mL CATSPER1 Ab, and a concentration of 20 µg/ml was selected for use in this study. Sperm were incubated with either (i) CATSPER1 Ab (20 µg/mL) (ii) caffeine (5 mM) (iii) CATSPER1 Ab (20 µg/mL) in combination with caffeine (5 mM) or (iv) with no treatment, for 10 min following which they were assessed for hyperactivation and rheotactic response. This experiment was replicated three times. Motility of the frozen-thawed sperm were analysed as described above.

Data Analysis

For gene expression data the formula E = 10^ (-1/slope)-1 was used where slope refers to the slope of the linear curve of cycle threshold (C_T) values plotted against log dilution. Only primers with PCR efficiencies between 90% and 110% were used. Gene expression was analysed using GenEx software (www.multid.se/genex.html) which allowed for compensation of PCR efficiencies, before averaging for RT-qPCR replicates. A normalisation factor, calculated based on the geometric mean of the two reference genes, GAPDH and ACTb, was used to normalise the expression of each gene of interest. Functional data were checked for normality of distribution, transformed where appropriate using a log transformation, and analysed using one-way Analysis of Variance (ANOVA), while qPCR data were analysed using univariate ANOVA in the Statistical Package for the Social Sciences (SPSS, Version 21.0; IBM, Armonk, NY, USA). Post-hoc tests were carried out using the Bonferroni correction and a P value < 0.05 was considered to be statistically significant.

Results

Bioinformatic Identification of Bovine *CATSPER* Orthologs

All four of the *CATSPER* genes reported in mice, humans, and horses were found to be present on chromosome 29, 21, 7, and 2, respectively in the bovine genome (Table 1). Multiple-sequence alignment was performed on novel *CATSPER* sequences with a complete second exon. Despite slight sequence variation between species, conserved areas were clearly identified across the three species for each protein (Fig. 1). Sequence similarity for CATSPER 1-4 peptides between bovine, murine and humans were 90, 85, 72 and 88%, respectively. Phylogenetic analysis showed that orthologous genes (identical by descent from the common ancestor of bovine, murine and human and in a conserved syntenic location) can be confidently predicted with bootstrap values generally in excess of 90% (Fig. 2). The phylogenetic relationships among different CATSPERs are much less certain. This is to be expected for genes that are so short that the phylogenetic signal is noisy and which are also known to be under selective pressure for different structure and function. A bootstrap value of 100 indicates that the sequences below that node consistently cluster together even with multiple resamplings of the data. The proteins are thus likely to be orthologs because their similarity is systemic and internally consistent rather than dependent on a few similar sites in the alignment.

Expression of *CATSPER 1-4* Genes along the Reproductive Tract of the Bull

The expression of all four *CATSPER* genes varied depending on the location along the reproductive tract of the bull. There was an effect of tissue location for expression of all four of the *CATSPER* genes, with *CATSPER1-4* upregulated in the parenchyma testis compared to the three segments of the epididymis ($P<0.01$; Fig 3). The rete testis had higher expression of all *CATSPER1-4* genes compared to the caudal and corpus epididymis ($P<0.01$), however, there was no difference in expression level between it and the caput epididymis or the parenchyma testis ($P>0.05$). *CATSPER1*

expression in the parenchyma testis was upregulated 6-fold, compared to the average of the housekeeping genes, which was the highest fold change of all the *CATSPER* genes. *CATSPER 2, 3* and *4* were upregulated 4.5-, 2.5- and 2.7-fold, respectively.

In Situ Immunocytochemistry for the CATSPER1 Protein

Fluorescence labelled to the principal piece was evident in frozen-thawed bull sperm stained with the anti-rabbit CATSPER 1 antibody (Fig. 4). CATSPER1 staining was primarily found along the principal piece of the flagella, however light staining was also found on the postacrosomal region. No staining was evident when the primary antibody was withheld (data not shown).

Effect of Extracellular Ca^{2+} on Hyperactivation and Rheotactic Response

There was no effect of EGTA treatment on the percentage of motile sperm. Caffeine increased hyperactivation but when extracellular calcium was removed by the addition of EGTA, there was a reduction in hyperactivation when compared to both the control and caffeine treated sperm ($P<0.001$). Interestingly, when caffeine was added back to the media containing EGTA, there was no increase in hyperactivation (Fig. 5A). A mirrored response was found for the rheotactic response, where caffeine significantly increased the number of sperm to progress along the channel, while chelating extracellular Ca^{2+} inhibited this behaviour ($P<0.01$). As before, adding caffeine back into the media did not result in a significant increase in rheotactic response (Fig. 5B). This data demonstrates that extracellular calcium is required for hyperactivation and rheotaxis of bull sperm. Treatment with EGTA or caffeine did not have an effect on the percentage of motile sperm.

Effect of Blocking Calcium Channels on Hyperactivation and Rheotactic Response

Similar to the removal of extracellular calcium above, there was no effect of blocking calcium channels on the percentage of motile sperm. However, incubation with caffeine increased sperm

hyperactivation while blocking calcium channels by pre-treatment with mibefradil prevented caffeine inducing hyperactivation ($P < 0.01$; Fig. 6A). A similar response was found with regards to the rheotactic response. Caffeine treatment increased sperm progression along the micro-channel, compared to the control group ($P < 0.01$; Fig 6B), however, when sperm were treated with mibefradil prior to caffeine, there was no significant increase in sperm progression ($P < 0.01$). This data demonstrates that an influx of calcium via mibefradil-sensitive channels is required for bull sperm hyperactivation and rheotaxis. Mibefradil treatment did not have an effect on the percentage motile sperm.

Effect of CatSper Channels on Hyperactivation and Rheotactic response

Blocking CatSper channels via CATSPER1 antibody reduced the levels of hyperactivity in the control sample and also inhibited the action of caffeine ($P < 0.01$; Fig. 7A). Similarly, CATSPER1 antibody reduced the ability of sperm to display rheotaxis compared to the control group and when added prior to caffeine it inhibited hyperactivation ($P < 0.01$; Fig. 7B). Despite the actions of both caffeine and the CATSPER1 antibody on hyperactivation and rheotactic response there was no effect of treatment on the percentage of motile sperm. Furthermore, sperm treated with CATSPER1 antibody displayed an increase in non-progressive motility compared to no treatment sperm and swam in anti-clockwise circles, as reported in CatSper null mice (Fig. 8, Supplemental Vid. S1 and S2.). This data demonstrates that an influx of extracellular calcium via CatSper channels is required for bull sperm hyperactivation and rheotaxis. Sperm treated with CATSPER1 antibody also displayed bending of the tail over the head.

Discussion

The advent of more completely annotated genomes from farm animal species is facilitating novel gene discovery at an unprecedented level. As genes that are required for the regulation of sperm motility, *CATSPER* hold significant promise in understanding the mechanisms by which sperm navigate to the site of fertilisation, but also in the design of new screening methods for diagnosing unexplained male infertility. The importance of *CATSPER* genes in male fertility has been clearly established, where disruption of the *CATSPER* genes leads to complete male infertility in both humans and mice [32, 33]. Despite fertility being an important issue in cattle, the role of CatSper channels in the bull has not been previously investigated. In this study, we show for the first time that *CATSPER 1-4* are expressed in the testes of the bull, where they have a role in sperm hyperactivation and rheotaxis.

CATSPER 1-4 are conserved in the *Bos taurus* genome. Using a comparative genomics approach, we searched the *Bos taurus* genome for homologs of the four *CATSPER* genes that have recently been described [8, 9, 19]. We found homologs of all four *CATSPER* subunit genes in the *Bos taurus* genome on various chromosomes, and revealed that the bovine *CATSPER* genes are present in similar synthetic sequence to those of humans and mice [9, 20]. Multiple-sequence alignment showed the conserved regions over the three species for each of the CATSPER protein sequences. Conserved regions highlight that the CATSPER proteins in *Bos taurus*, *Homo sapiens* and *Mus musculus* have an evolutionary relationship by which they share a lineage and are descended from a common ancestor. Phylogenetic analysis of these bovine genes in conjunction with their human and mouse orthologs showed a high degree of sequence similarity, which suggests functional conservation of these genes over the course of evolution. Where both a first and a second exon could be recovered for the bovine ortholog, the percentage of sequence identity ranged from 72% (*CATSPER3*) to 90% (*CATSPER1*). The high degree of similarity of the bovine genes to their human

and mouse orthologs prompted us to delve further into the specific expression and function of CatSper channels in bull sperm.

Expression analysis at the gene level showed that *CATSPER1-4* mRNA was expressed in the reproductive tract of the bull with highest expression in the parenchyma testis, indicating that they are incorporated into sperm during spermatogenesis. Of the four *CATSPER* genes, *CATSPER1* was found to have the highest expression across all tissue segments and is in agreement with studies in mice and humans [19, 33]. The expression of *CATSPER1* was twofold that of *CATSPER3* and *4* in the parenchyma testis. This is of particular interest as recently the profiles of *CATSPER1* mRNA expression in testis biopsy of subfertile human male patients were investigated [34]. Compared with patients whose infertility cannot be ascribed to a deficiency in motility, a significant reduction in the level of *CATSPER1* gene expression among patients with asthenospermia was observed leading the authors to propose that *CATSPER1* expression be used as a non-invasive screening method for male infertility [35]. Additionally, *CATSPER1*-deficient mice are infertile as a result of an impairment of sperm motility and an inability to fertilise oocytes [18]. Immunocytochemical analysis of the CATSPER1 protein revealed CATSPER1 to be localized to the principal piece of the flagellum which is in agreement with the staining pattern reported for mouse, human and stallions [8, 18, 19]. This same expression and localization of CATSPER1 in bulls as in humans, mice and stallions points to a similar role of CATSPER1 all species.

Hyperactivation in sperm of other species is dependent upon the presence of the cation channel CatSper. Therefore, we investigated the function of this channel in bull sperm. We found that caffeine was an effective inducer of hyperactivation in bull sperm, as has been reported in other studies and thus was used as a positive control for all functional studies [30, 36]. While caffeine is known to induce hyperactivation in both capacitated and non-capacitated bull sperm by inhibiting phosphodiesterase and thus increasing cAMP [37], studies have also shown that caffeine induced hyperactivation, requires exracellular Ca^{2+} in bull sperm [30, 36] which is in agreement with this

study as sperm treated with caffeine did not hyperactivate while in Ca^{2+}-deficient medium. We investigated if bull sperm require extracellular Ca^{2+} for hyperactivated motility and the ability to display a sufficient rheotactic response to fluid flow. The results from this study, in which EGTA was used to chelate calcium, indicate that bull sperm require extracellular calcium for both hyperactivation and rheotactic responses. While the need for extracellular calcium for hyperactivation has been reported, this is the first study to our knowledge to report that bull sperm requires extracellular Ca^{2+} to effectively induce rheotaxis. The typical characteristics of bull sperm hyperactivation, when assessed in a static droplet, is an increase in swimming speed of sperm but a non-progressive fashion, however, although not assessed directly in this study, it appears that when hyperactivated bull sperm are exposed to a fluid flow they are able to maintain the increase in swimming speed but alter their swimming pattern to linear.

The T-type calcium channel blocker mibefradil has been reported to block calcium currents in stallion and human sperm including that mediated by CatSper [8, 38]. Treatment of equine sperm with mibefradil, has been shown to reduce the influx of Ca^{2+} associated with intracellular alkalinization [8]. We found similar results in this study, where treatment of bull sperm with mibefradil reduced both percentage hyperactivation and rheotactic response. These results tell us that calcium channels are required for both of these sperm functions. Although CatSper channels are T-type calcium channels, the lack of specificity of mibefradil to CatSper channels alone led us to assess hyperactivation and rheotaxis following blocking with a CATSPER1 antibody, to target the CatSper channel specifically. Treatment with CATSPER1 antibody significantly inhibited the increase in hyperactivation and rheotaxis when compared to the caffeine treatment group. Interestingly, we found that with antibody treatment alone there was a decrease in both hyperactivation and rheotactic response to that below the level of the no treatment control. This tells us that blocking of the CatSper channels has a negative effect on sperm function, independent from the effect of caffeine. Mouse sperm lacking CatSper induced Ca^{2+} influx tend to swim in an

anticlockwise circular plane, which does not change regardless of fluid flow [3]. We observed a similar swimming pattern in the bull sperm treated with CATSPER1 antibody. We observed a small proportion of sperm that had been treated with CATSPER1 Ab displayed bending of the tail over the sperm head. The reason for this is unknown. None of the calcium blocking treatments influenced the percentage of motile sperm, allowing us to separate the effect of diminished hyperactivation and rheotactic response from that of changes in motility. Thus, our findings suggest that hyperactivation and rheotactic response is mediated by CatSper channels.

In conclusion, this study is the first to identify and characterise *CATSPER* genes in the bull. The testes expression and location-specific changes in mRNA abundance support an evolutionary conserved role for these channels in bull reproduction. This study demonstrates that CatSper channels play a critical role in hyperactivation and rheotactic response in bull sperm. The location and functionality of these channels points to their use as potential male infertility markers and should provide a basis for much future research to define the factors associated with effective sperm–oocyte interactions in this species.

Acknowledgment

The authors gratefully acknowledge the financial support from the National Cattle Breeding Centre (Naas, Co Kildare, Ireland) for the donation of bull semen.

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Table Legends

Table 1. Oligonucleotide sequences for primers as well as the genomic coordinates of the *CATSPER* genes (UCSC version bosTau8).

459 **Figures Legends**

460 **FIG. 1.** Multiple sequence alignment of CATSPER1-4 (A-D) proteins in bovine, human and mouse
461 showing conserved regions (highlighted in colour). Conserved regions highlight that the CATSPER
462 proteins in *Bos taurus*, *Homo sapiens* and *Mus musculus* have an evolutionary relationship by which
463 they share a lineage and are descended from a common ancestor.

464

465 **FIG. 2.** Phylogenetic tree showing the evolutionary relationship between the between the four
466 *CATSPER* genes in the bovine genome and their human and mouse orthologs. The numbers at the
467 nodes are % bootstrap values.

468

469 **FIG. 3.** Expression of four selected *CATSPER* genes across the reproductive tracts of three mature
470 bulls. Expression was normalised to the average gene expression of both *GAPDH* and *ACTβ*. Tissue
471 sections analysed were; parenchyma testis, rete testis, caput epididymis (caput), corpus epididymis
472 (corpus), and cauda epididymis (cauda). Values are means ± SEM for three independent replicates.
473 ^{abcd} Different superscripts differ significantly within each gene ($P < 0.05$).

474

475 **FIG. 4.** Immunostaining for CATSPER1 (green) and nuclei counterstained with DAPI (blue; A).
476 Fluorescent imaging showing co-localisation of CATSPER1 to bull sperm flagellum. CatSper1 is
477 specifically labeled in the principal piece of the tail (B). Bar = 20 μm.

478

479 **FIG. 5.** The effect of removing extracellular calcium using ethylene glycol tetraacetic acid (EGTA)
480 treatment on (A) hyperactivation and (B) rheotactic response of bull sperm. Number of sperm refers

to the number of sperm to swim past the 10 mm mark of the microfluidic channel. Values are means ± SEM for three independent replicates. [abcd] different superscripts differ significantly within panel ($P < 0.05$).

FIG. 6. The effect of Mibefradil treatment on (A) hyperactivation and (B) rheotactic response of bull sperm. Number of sperm refers to the number of sperm to swim past the 10 mm mark of the microfluidic channel. Values are means ± SEM for three independent replicates. [abcd] different superscripts differ significantly within panel ($P < 0.05$).

FIG. 7. The effect of CATSPER1 antibody on (A) hyperactivation and (B) rheotactic response of bull sperm. Number of sperm refers to the number of sperm to swim past the 10 mm mark of the microfluidic channel. Values are means ± SEM for three independent replicates. [abcd] different superscripts differ significantly with panel ($P < 0.05$).

FIG. 8. Stills form supplementary videos showing the swimming patterns of control (A) and sperm treated with CATSPER1 antibody (B). Sperm swimming in an anticlockwise direction (circle) and bending of the sperm tail over the head (arrows) was observed in the Ab treated group (B).

Supplemental Data Legends

Supplemental Vid. S1.

Video showing normal motility of control sperm. Sperm are observed to be swimming in a progressive manner.

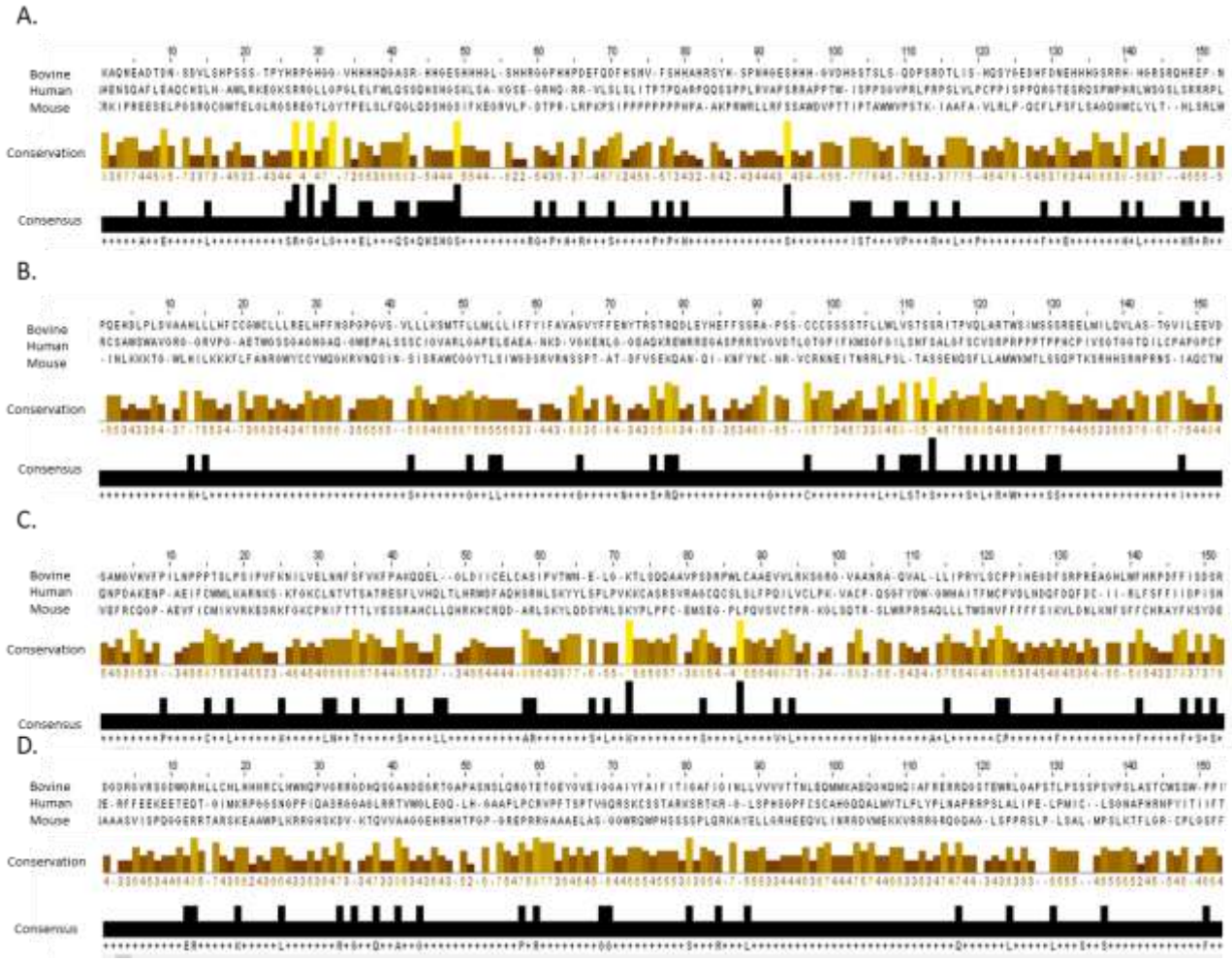
Supplemental Vid. S12

Sperm treated with 20 µg/mL CATSPER1 antibody swim in an anticlockwise direction (blue circle). Bending of the sperm tail over the head of sperm was observed (arrows).

Tables

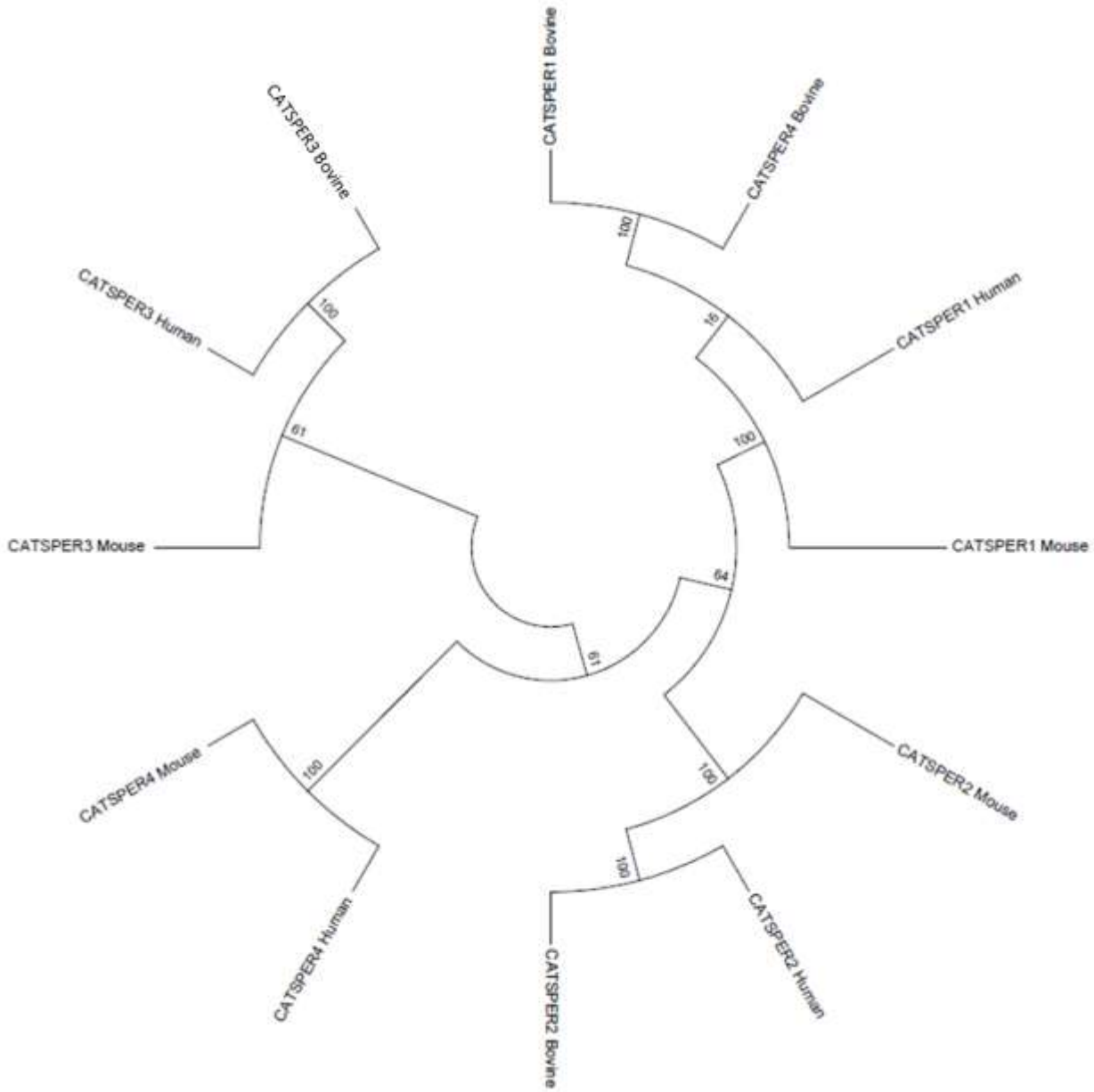
Gene symbol	Forward primer (5'- 3')	Reverse primer (5'- 3')	Genomic Co-ordinates
CATSPER1	TACTCTGACCCCAAACGCTT	GGCTGTCCAGGTAGATGAGG	chr29:44,771,514-44,779,197
CATSPER2	CCTCAAGAGCATGACCTTCC	GCGAGTTGAACGGGTGTAAT	chr21:55,906,680-55,924,768
CATSPER3	GACTCTGCCTGGGTTTCTCT	CACATCGAGCAGGTTGTAGC	chr7:48,012,507-48,043,848
CATSPER4	GACGGAGACAGGGGAGTAC	TGAAGGCTATTTGATGCTGGTG	chr2:127,488,171-127,499,030

Figures



508

509 Figure 1



510

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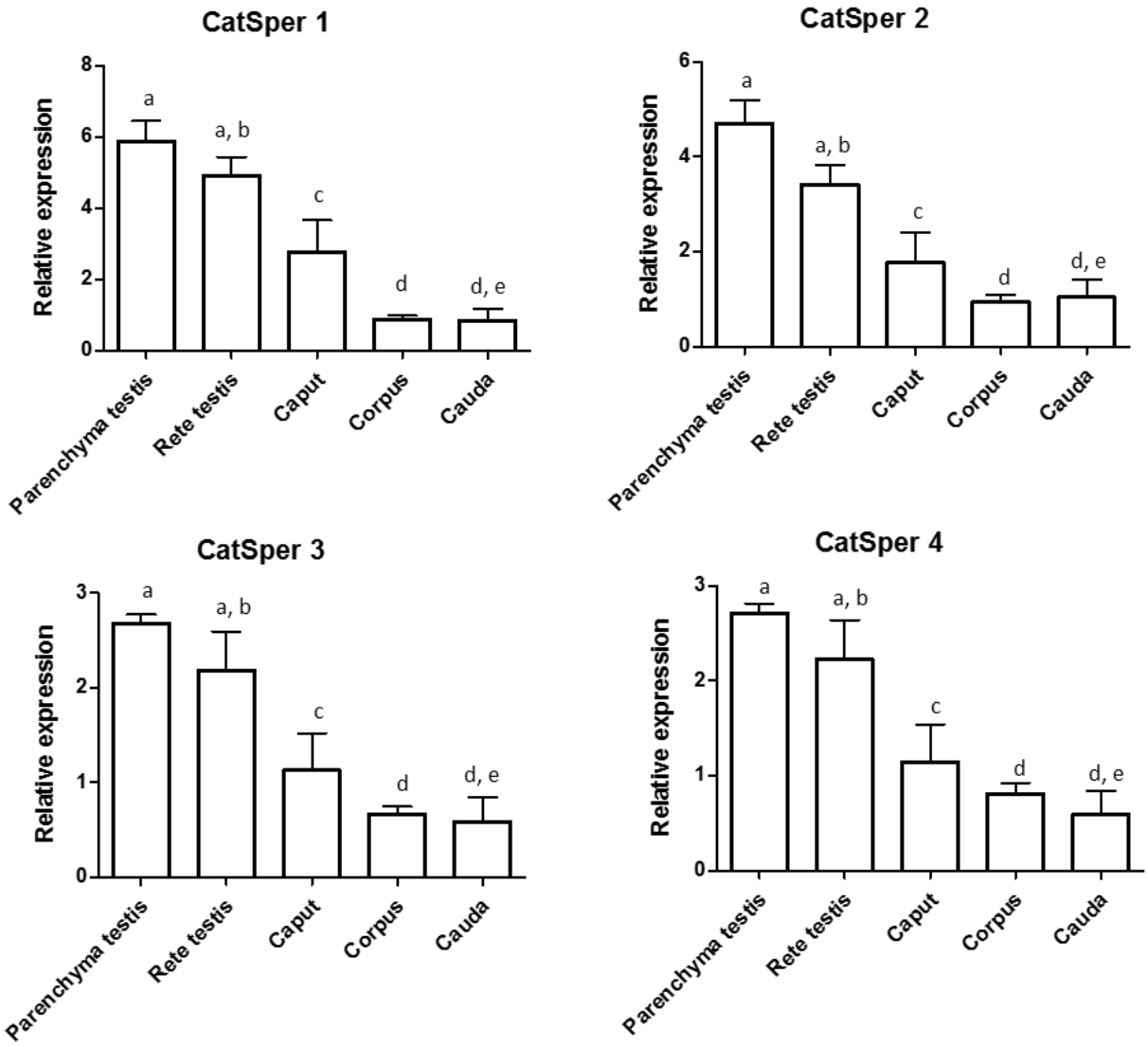

512

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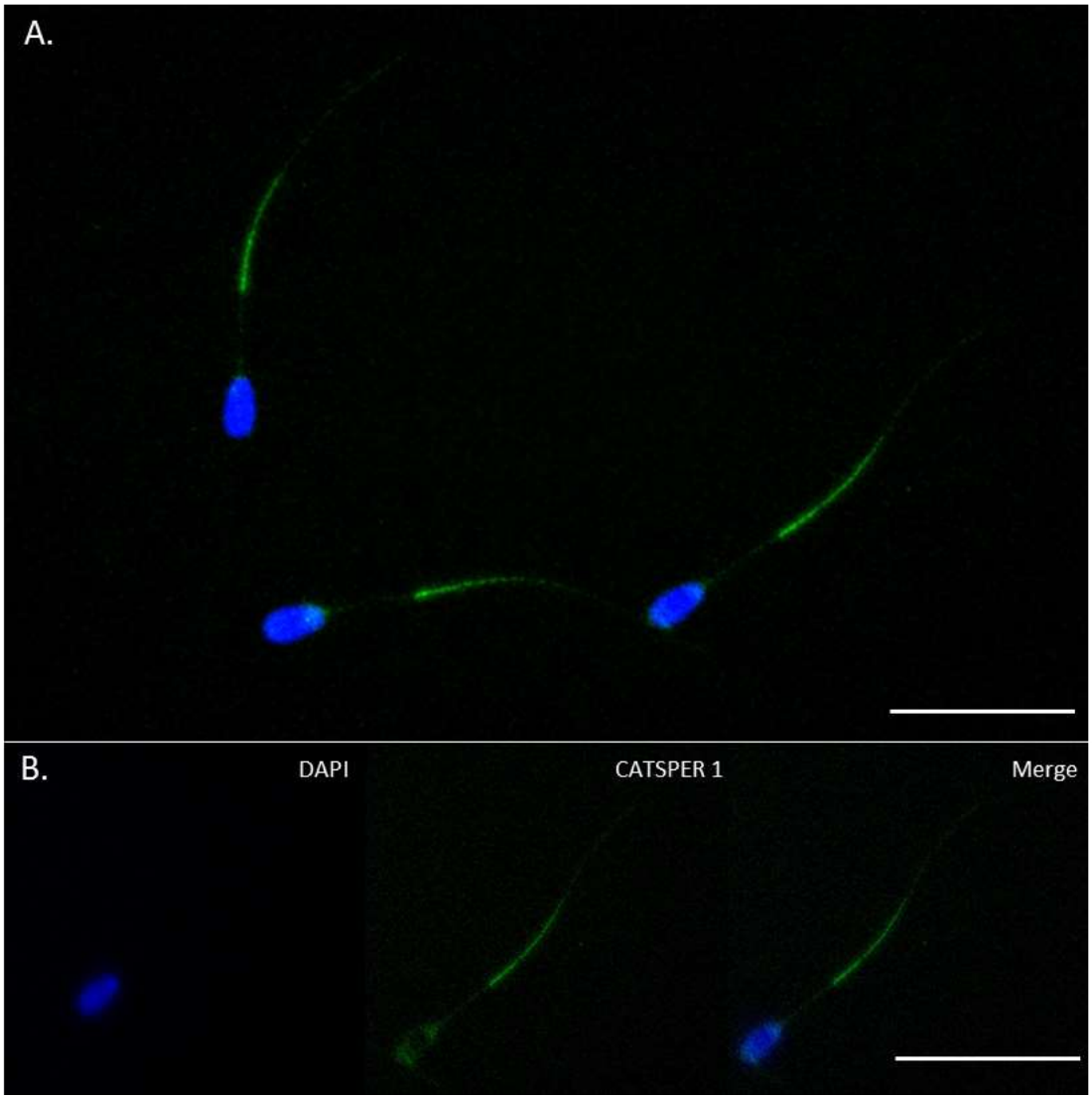

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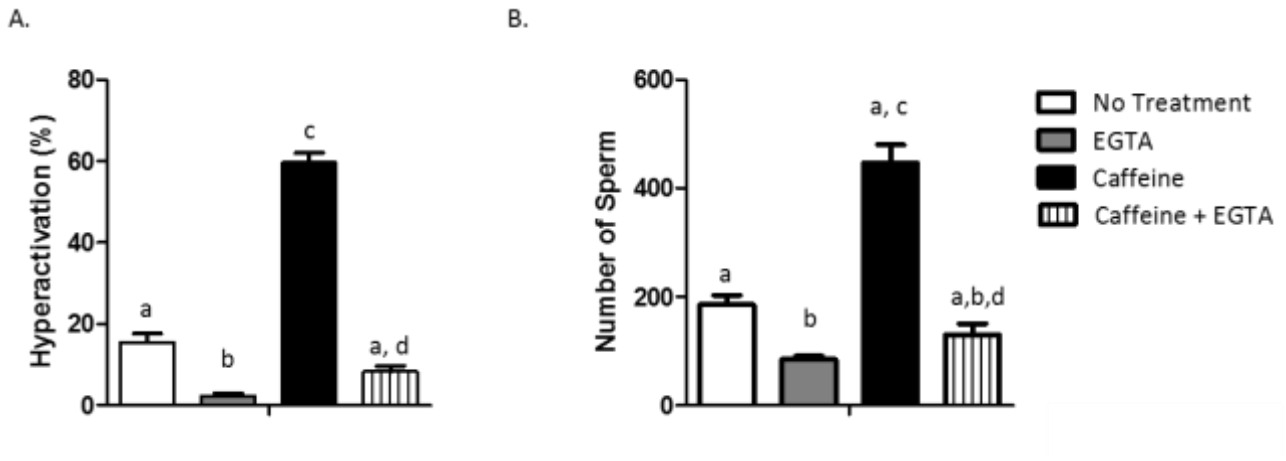

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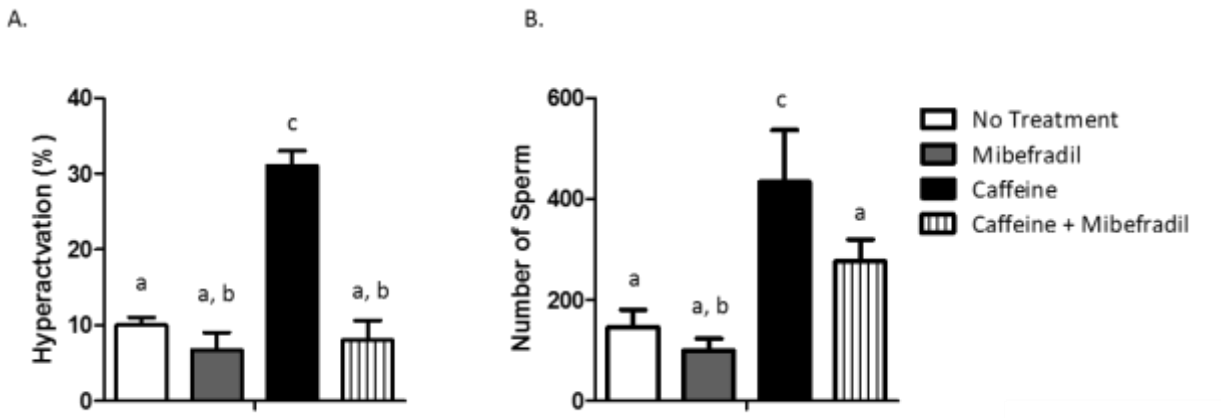
516 Figure 5



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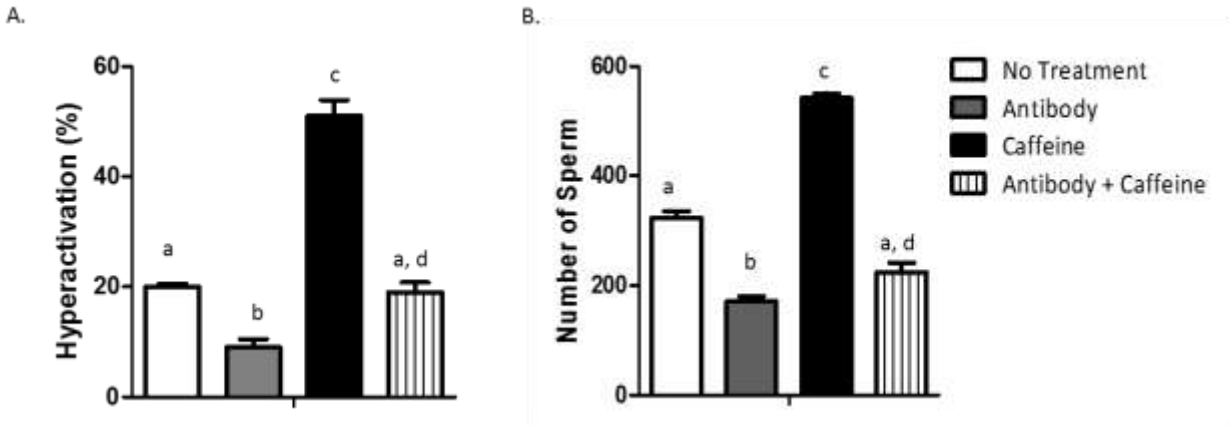
518 Figure 6



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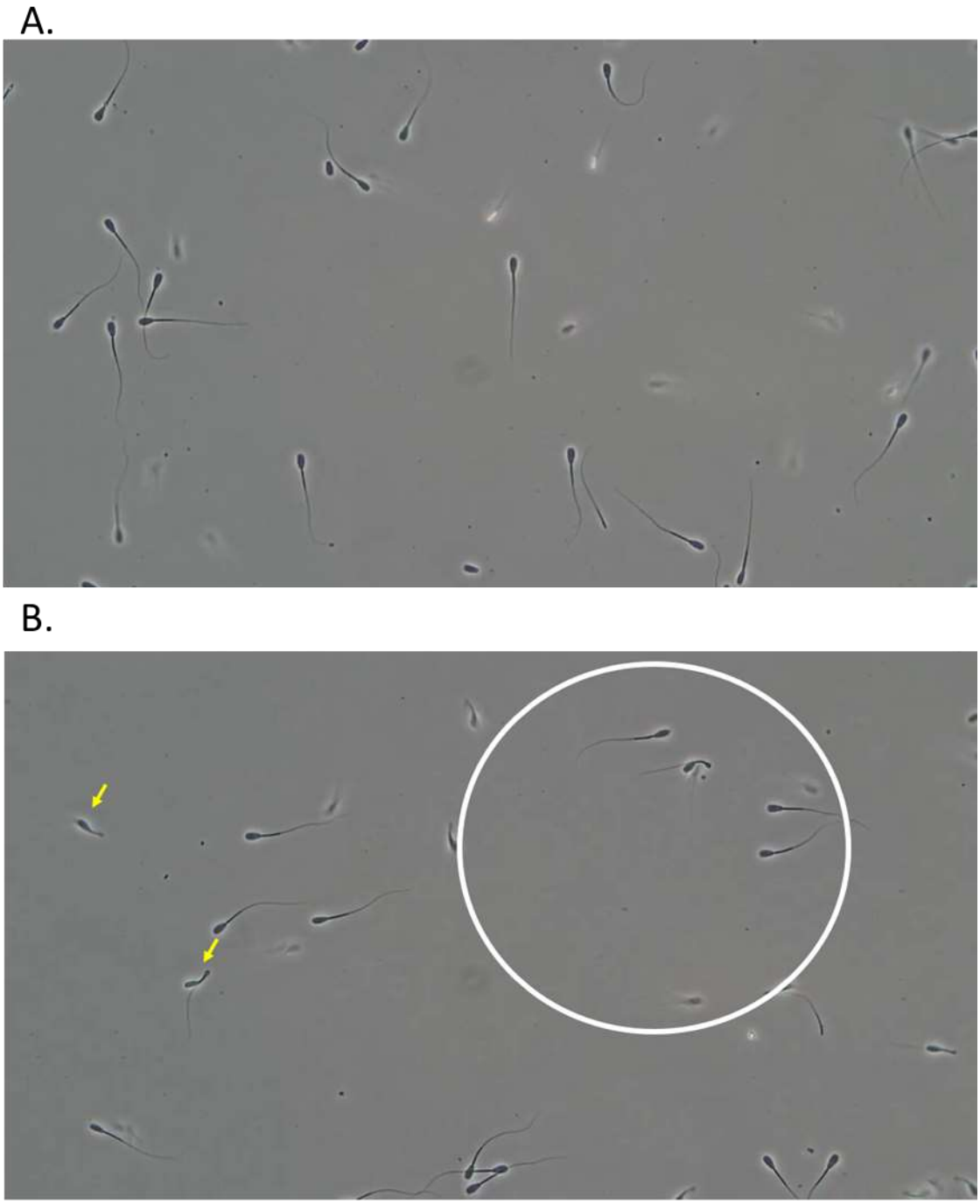
520 Figure 7



521

522 Figure 8